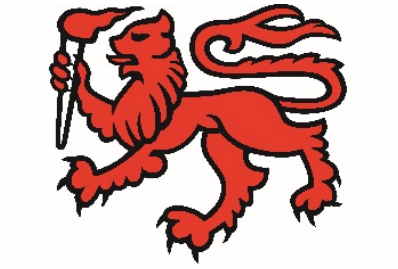


UNIVERSITY*of*
TASMANIA

The regional lung response to mechanical ventilation

By

Seiha Yen

BSc (Hons)

School of Medicine, College of Health

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania June 2019

Statements and declarations

Declaration of originality:

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

_____

Date 28th May 2019

Seiha Yen

PhD Candidate

_____

Date 28th May 2019

Professor Graeme Zosky

Principal Supervisor

Authority of access:

The publishers of the paper in Chapter 2 hold the copyright for that content and access to the material should be sought from the Journal. The remaining non-published content of the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

_____

Date 28th May 2019

Seiha Yen

PhD Candidate

_____

Date 28th May 2019

Professor Graeme Zosky

Principal Supervisor

Statement of Co-Authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Candidate: Seiha Yen, School of Medicine, University of Tasmania

Author 1 (Supervisor): Graeme Zosky, School of Medicine and Menzies Institute for Medical Research, College of Health and Medicine, University of Tasmania, Hobart, Australia

Author 2: Melissa Preissner, Department of Mechanical and Aerospace Engineering, Monash University, Melbourne, Victoria, Australia

Author 3: Ellen Bennett, School of Medicine, College of Health and Medicine, University of Tasmania, Hobart, Australia

Author 4: Peter Dargaville, Menzies Institute for Medical Research, College of Health and Medicine, University of Tasmania, Hobart, Australia

Author 5: Louise Roddam, School of Medicine, College of Health and Medicine, University of Tasmania, Hobart, Australia

Author 6: Ronan O'Toole, School of Medicine, College of Health and Medicine, University of Tasmania, Hobart, Australia

Author 7: Stephen Dubsky, Department of Mechanical and Aerospace Engineering, Monash University, Melbourne, Victoria, Australia

Author 8: Andreas Fouras, 4Dx Limited, Melbourne, Victoria, Australia

Author 9: Richard Carnibella, 4Dx Limited, Melbourne, Victoria, Australia

Author 10: Heather Jones, Biomedical Imaging Research Institute, Department of Medicine, and Women's Guild Lung Institute, Cedars-Sinai Medical Center, Los Angeles, California

Author 11: Rhiannon Murrie, Department of Mechanical and Aerospace Engineering, Monash University, Melbourne, Victoria, Australia

Author details and their roles:

Paper 1 - The link between regional tidal stretch and lung injury during mechanical ventilation (published in *American Journal of Respiratory Cell and Molecular Biology*, 2019; 60: 569-577)**:**

Located in Chapter 2

Candidate was the primary author and contributed to the conception and design of the research project (with authors 1, 2, 4, 5, 6, 7, 8 and 10), conducted the experiments (with authors 1, 2, 3, 7, 9 and 10), analysed the data (with authors 1, 2, 3, 4, 7, 8, 9 and 10) and drafted significant parts of the paper in consultation with all other authors.

Paper 2 - The association between regional lung volumes and gene expression during mechanical ventilation with endotoxemia (currently under review with *American Journal of Physiology – Lung Cell and Molecular Physiology*)

Located in Chapter 3

Candidate was the primary author and contributed to the conception and design of the research project (with authors 1, 2, 4, 7 and 8), conducted the experiments (with authors 1, 2, 3, 7, 9 and 11), analysed the data (with authors 1, 2, 3, 4, 7, 8, 9 and 11) and drafted significant parts of the paper in consultation with all other authors.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed:

Professor Graeme Zosky	Professor Lisa Foa
Supervisor	(Acting) Head of School
School of Medicine	School of Medicine
University of Tasmania	University of Tasmania
Date: 28/05/2019	28/05/2019

Statement of ethical conduct:

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University of Tasmania.

_____

Date 28th May 2019

Seiha Yen

PhD Candidate

_____

Date 28th May 2019

Professor Graeme Zosky

Principal Supervisor

Acknowledgements

I would like to express my special thanks to all the people who helped me along the way to completing my Thesis. First of all, my supervisors: I would like to thank Professor Graeme Zosky for giving me the opportunity to undertake this study and for your support in guiding me throughout my time here, especially during difficult situations. Thanks to Dr Ronan O'Toole for introducing me to Professor Graeme Zosky, for agreeing to be my co-supervisor, and for your contribution to the project and to Dr Louise Roddam for being my co-supervisor and for your contribution to the project.

I would like to thank everyone in the Lung Growth and Environmental Health team. Thanks to Dr Ellen Bennett for all your assistance throughout my time here, especially for your contribution and help in preparation for experiments. Thanks to Lewis William, Dr Ling Chen, Dr Naile Nunez, Ryan Giblin, Santon Thaver, Isobel Rogers and Ella Smalley for your support and for making my time here enjoyable.

Thank you to the project collaborators: To Professor Peter Dargaville, thank you for your time and contribution, especially in the development of the project and the manuscripts. Thanks to Professor Andreas Fouras, Melissa Preissner, Dr Richard Carnibella, Dr Heather Jones, Dr Stephen Dubsky and Dr Rhiannon Murrie for your contribution to project development, undertaking the ventilation and lung imaging experiments and imaging analyses.

To the University of Tasmania Graduate Research Office and School of Medicine, for accepting my enrolment and providing me with a PhD scholarship. Also, thanks to my Graduate Research Co-ordinators, Dr Stephen Richard and Dr Bruce Lyon, for your time and advice.

A big thank you to my family: My parents, brothers and sisters, who have given me great support from afar. To my wife and kids, thank you for making everyday life enjoyable. To Cambodians in Hobart for being part of our small but friendly community and sharing our time as a big extended family.

Table of contents

List of figures

List of tables

Abbreviations

4D CT	Four-dimensional computed tomography
AECC	American-European Consensus Conference
ALI	Acute lung injury
Ang-2	Angiopoietin-2
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
ARDSNet	ARDS network study
BAL	Bronchoalveolar lavage
Ccl2	Chemokine (C-C motif) ligand 2 also refer to MCP-1
Cdh1	E-cadherin
c-fos	FBJ Osteosarcoma oncogene
C_T	Cycle threshold
CT	Computed tomography
Ctnnb1	β-catenin
CxCl2	Chemokine (C-X-C motif) ligand 2 also known as MIP-2
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Egfr	Epidermal growth factor receptor

Elane	Neutrophil elastase
ELISA	Enzyme link immunoassay
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FiO2	Fraction of inspired oxygen
FRC	Functional residual capacity
HSR	Haemorrhagic shock-resuscitation
HU	Hounsfield Units
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IP	Intraperitoneal
IT	Intra-tracheally
LPS	Lipopolysaccharide
Mapk	Mitogen-activated protein kinase

MCP-1	Monocyte Chemoattractant Protein-1
MIP-2	macrophage inflammatory protein-2
MPO	Neutrophil myeloperoxidase
MRI	Magnetic resonance imaging
MSOF	Multisystem organ failure
Nfe2l2	Nuclear factor erythroid-derived 2-like 2
NF-κB	Nuclear factor kappa B
PA	Plasminogen activator
$PaCO_2$	Partial pressure of carbon dioxide
PaO_2	Partial pressure of oxygen
PCR	Polymerase chain reaction
PEEP	Positive end expiratory pressure
PET	Positron emission tomography
PIP	Peak inspiratory pressure
Plat	Tissue type plasminogen activator
qPCR	Quantitative polymerase chain reaction
RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic acid
Rpl37	Gene encode 60S ribosomal protein L37
SD	Standard deviation

sFRC	Specific functional residual capacity (regional FRC/regional lung volume)
Sftpb	surfactant protein B
sVT	Specific tidal volume (regional VT/regional lung volume)
Tgfb1	Transforming growth factor β1
TNF-α	Tumour necrosis factor-alpha
VILI	Ventilator-induced lung injury
Vim	Vimentin
V_T	Tidal volume
Wnt	Wingless integration
ZEEP	Zero PEEP

Thesis abstract

Mechanical ventilation is a lifesaving therapy for patients with acute respiratory failure. Unfortunately, mechanical ventilation can contribute to mortality in these critically ill patients through a process known as ventilator-induced lung injury (VILI). VILI is thought to occur through overdistension and/or cyclic stretch of the lung which leads to tissue damage and inflammation (biotrauma). However, due to the heterogenous response of the lung to mechanical ventilation, both overdistension due to high lung volume ventilation and cyclic stretch due to low lung volume ventilation can occur simultaneously within the same lung. In addition, the magnitude of VILI is influenced by the pre-existing injury that caused respiratory failure in the first instance. To date, the link between variation in the regional lung volume response and regional lung inflammation, and how different lung injuries alters these responses, is poorly understood. The aim of this Thesis was to assess the impact of mechanical ventilation on the regional lung volume response and biotrauma in the healthy, indirectly injured (endotoxemia) and directly injured (acid aspiration) lung.

To assess the impact of mechanical ventilation on the regional response in the healthy lung (Chapter 2), adult female BALB/c mice were ventilated for 2 hours using either a protective (moderate PIP with PEEP) or injurious (high PIP with no PEEP) ventilation strategy. The regional FRC (the regional volume of air retained in the lung at end expiration) and tidal volume responses were obtained from analysis of dynamic high-resolution 4D CT lung images at baseline and after 2 hours of ventilation. The expression of 21 genes were measured by qPCR array and correlated with regional lung volume responses. Protein expression and neutrophilia were quantified by immunohistochemistry. To assess the effect of indirect lung injury on the response (Chapter 3), mice were exposed to lipopolysaccharide (LPS) by IP injection in saline

or saline alone. Four hours later, lungs were imaged by 4D CT at baseline and after 2 hours of ventilation. The inflammatory response was evaluated by the expression of seven inflammatory genes by qPCR and neutrophilia was quantified by immunohistochemistry. To assess the effect of direct lung injury (Chapter 4), mice were exposed intra-tracheally (IT) to hydrochloric acid or saline. Lungs were imaged by 4D CT at baseline and after 2 hours of ventilation and the regional expression of four inflammatory cytokines was assessed by ELISA.

In the healthy lung, there was regional variation in the lung volume response and gene expression, whereby regional tidal volume, and the expression of two genes (*IL-6*, $P = 0.02$ and *Ccl2*, $P < 0.01$), varied regionally depending on the ventilation strategy. The expression of *IL-6* and *Ccl2* was positively associated with regional tidal volume, but not with FRC, suggesting that overstretch is detrimental in the healthy lung. In endotoxemia (LPS exposure), there was also regional variation in FRC and the expression of the two inflammatory genes (*IL-6* and *Ccl2*). The expression of *IL-6* was negatively associated with FRC in the LPS treated mice, whereas there was no significant association between tidal volume suggesting that the endotoxemic lung is susceptible to low lung volume ventilation. In contrast, acid aspiration had no effect on regional FRC or regional tidal volume. There was also no effect on regional levels of the inflammatory cytokines (TNF-α, MCP-1, IL-1β and IL-6). Further analysis to assess the effect of IT fluid administration on the response was undertaken by comparing lung volume data from the IP saline exposed group (Chapter 3) with the saline IT exposed mice (Chapter 4). Saline exposure via the IT route led to significant increases in FRC in some regions ($P = 0.01$).

This Thesis showed that healthy lung is susceptible to regional overdistension, while the endotoxemic lung is susceptible to low lung volume ventilation. In both cases,

these were associated with increased expression of *IL-6*. Unfortunately, pre-injury in the acid aspiration model was not established, which may be due to the high pH used, short exposure duration and the impact of liquid aspiration. Therefore, it is unclear whether this injury alters the regional stretch and inflammatory response compared to endotoxemia and further experiments are required. Overall, the data from these experiments provide critical insight into the regional lung response to mechanical ventilation and the influence of existing injury on the response. In particular, these results highlight the importance of the balance between cyclic stretch and over-stretch (tidal volume) and how each of these can contribute to lung inflammation and, potentially, patient outcomes.

Thesis structure

This thesis consists of five chapters

- Chapter 1 is the Thesis Introduction and consists of a literature review, aims and hypotheses and a brief description of the study approach used to address each specific aim.

- Chapter 2 is a results Chapter that addresses the first Aim of the study; the association between regional lung volumes and the expression of VILI associated genes in response to mechanical ventilation in the healthy lung. This Chapter is presented as published in the *American Journal of Respiratory Cell and Molecular Biology*.

- Chapter 3 is a results Chapter that addresses the second Aim of this study; the impact of endotoxemia, as a model of indirect lung injury, on the regional lung volume and gene expression response to mechanical ventilation. This Chapter is formatted as submitted to the *American Journal of Physiology – Lung Cell and Molecular Physiology* (currently under review).

- Chapter 4 is a results Chapter that that addresses the third Aim; the impact of acid aspiration, as a model of direct lung injury, on regional lung volumes and the expression of VILI-related protein in response to mechanical ventilation.

- Chapter 5 is the General Discussion and outline the Conclusions of the Thesis.

Chapter 1 - Literature review

1.1 Overview of respiratory failure and mechanical ventilation

The lung plays a critical role in the exchange of oxygen and carbon dioxide. This exchange process consists of ventilation, which involves the inhalation and exhalation of air between the lungs and the environment, and gas exchange, which occurs within the lungs between the alveoli and the capillaries. Many pathological circumstances can lead to the impairment of gas exchange and/or ventilation, such that the lung is not able to support normal physiological function [1, 2] resulting in respiratory failure. Respiratory failure may be characterised by hypoxemia due to impairment of gas exchange, as indicated by a partial pressure of oxygen (PaO_2) in arterial blood (< 60 mm Hg), with or without ventilation impairment leading to hypercapnia ($PaCO_2$ > 45 mm Hg) [1, 2]. Acute Respiratory Distress Syndrome (ARDS) is a severe form of respiratory failure and has a very high mortality rate [3-5].

The clinical care of patients with acute respiratory failure involves securing the airway and management of breathing and circulation which often includes mechanical respiratory support [1]. Invasive mechanical ventilation is used as a lifesaving respiratory support therapy for patients with acute respiratory failure including patients with ARDS [6]. Unfortunately, mechanical ventilation is thought to contribute to the development of ARDS and its high mortality by inducing inflammation and contributing to multiple system organ failure (MSOF) [7, 8]. Mechanical ventilation is thought to cause injury by a process known as ventilator-induced lung injury (VILI) [9]. VILI may develop as a result of overdistension due to high tidal volumes and/or atelectrauma due to cyclic opening and closing of the airways and recruitment/de-recruitment of alveoli as a result of low end-tidal lung volumes [10-12]. This suggests a balance is required whereby the lung is adequately ventilated to support life while avoiding excessively high and low lung volumes. As a result, many studies have

focussed on finding the ideal ventilation strategy that provides sufficient gas exchange but does not cause injury to the lungs [6]. However, this is complicated by the fact that the lung has a heterogeneous response to mechanical ventilation, with both overdistension and atelectrauma occurring within different regions of the same lung [13-15]. Minimising VILI in patients with ARDS is even more complicated because ARDS is a heterogenous disease with different causes and pathologies [16, 17], so the optimum ventilation strategy may vary between patients. The "two-hit" hypothesis suggests a patient's outcome is the result of the interaction between the primary disorder that caused respiratory failure and the lung response to mechanical ventilation [18]. The following sections will outline our current understanding of ARDS, the mechanisms of VILI, how they interact, and their impacts on a patient's outcome.

1.2 Acute respiratory distress syndrome (ARDS)

1.2.1 What is ARDS?

ARDS is a clinical syndrome characterised by inflammation in the lung manifested by pulmonary oedema and flooding of the alveolar space resulting in loss of lung compliance and hypoxemia [19]. Initially, Ashbaugh and colleagues [20] used the term Respiratory Distress Syndrome to describe the clinical condition of 12 adult patients who exhibited acute onset tachypnoea (rapid breathing), hypoxemia (oxygen deficiency), loss of lung compliance, and diffuse alveolar infiltration seen on chest X-ray. Over time, several new terms and characteristic criteria have been introduced to describe these conditions [21]. At the 1994 American-European Consensus Conference (AECC) on ARDS, the terms Acute Lung Injury (ALI) and ARDS were defined to characterise alveolar leakage, oedema formation, pulmonary epithelial cell death, loss of lung compliance, hypoxemia, and bilateral infiltrates on chest radiograph [22]. ALI and ARDS were differentiated on the basis of arterial blood oxygenation:

$PaO_2/FiO_2 \leq$ 200 mm Hg for ARDS and 200 mm Hg < $PaO_2/FiO_2 \leq$ 300 mm Hg for ALI. Recently, the ARDS Definition Task Force developed the "Berlin Definition", where the term ALI was dropped and three mutually exclusive categories of ARDS were defined based on the degree of hypoxemia: mild (200 mm Hg < $PaO_2/FiO_2 \leq$ 300 mm Hg), moderate (100 mm Hg < $PaO_2/FiO_2 \leq$ 200 mm Hg), and severe ($PaO_2/FiO_2 \leq$ 100 mm Hg) [4].

The development of ARDS is associated with a variety of clinical disorders which can be classified into direct and indirect injuries to the lung [21]. Sepsis is the most common cause of indirect injury, while pneumonia and aspiration are the most common causes of direct injury [21]. The relative contribution of these causes of ARDS to the disease burden varies between studies. For example, sepsis was found to cause the most ARDS cases in Europe and the USA between the 1990s to 2000s [21, 23-26], while, pneumonia was the most common cause of ARDS in Taiwan [27] and Brazil [28] in more recent studies. In contrast, malaria, leptospirosis, and undiagnosed fever followed by pneumonia were found as the main causes of ARDS in a recent study in India [29]. These variations are important as the cause of ARDS can be a determinant of disease severity, mortality and treatment. Mortality rates due to ARDS vary according to the cause of the initial injury and disease severity. Patients with pulmonary sepsis or aspiration, two common causes of ARDS, have the highest mortality rates [23, 24]. As you would expect, patients with mild ARDS typically have a lower mortality rate than patients with moderate or severe ARDS [23, 24, 27, 28, 30].

1.2.2 Incidence of ARDS and ARDS-related mortality

Studies report a wide range of annual incidences of ARDS. A prospective, population-based cohort study in King County, Washington (U.S.A.), between April 1999 and

July 2000, the annual incidence of ARDS was estimated 78.9/100,000 people with 26% of them being classified as ALI (mild ARDS) [23]. Based on this calculation the annual incidence of ARDS was estimated to be about 200,000 in the USA [23]. In contrast, a lower annual ARDS incidence rate (15.74/100,000 people) was reported from a 15-year nationwide inpatient cohort study based on the Taiwan National Health Insurance Research Database between 1997 to 2011 [27]. This discrepancy may reflect the different diagnosis methods and selection criteria used in these two studies [27], while misdiagnosis is also a major issue [5].

Overall ARDS mortality is high and has remained unchanged for a number of decades. For example, while the U.S. study mentioned earlier [23] reported mortality rates of 38.5% for mild ARDS and 41.1% for moderate and severe ARDS, the inpatient cohort study in Taiwan [27] reported a mortality rate of 57.8%. Meanwhile the ARDS Definition Task Force performed meta-analysis of 4457 patients with ARDS from four multi-centre and three single-centre clinical studies and found mortality rates due to mild, moderate, and severe ARDS, were 27, 32 and 45% respectively [4]. A recent prospective study found a mortality rate of approximately 40% in patients with ARDS in 459 ICU across 50 countries worldwide [5]. A systematic analysis of 72 ARDS studies, with at least 30 patients, between 1994 to 2005 found an overall mortality rate due to ARDS of 43% with an annual reduction of 1.1% based on regression equations [31]. In contrast, another systematic review reported the mortality rates did not decrease between 1994 and 2006 [32]. Recent systematic reviews have shown that the mortality rates due to ARDS remains high (over 40%) [3, 33], highlighting the poor outcomes in these patients.

1.2.3 ARDS and inflammation

ARDS is associated with high levels of inflammatory cells and cytokines both locally (in the lung) and systemically (in blood and peripheral organs). In early studies, excessive numbers of neutrophils and high levels of elastase were found in the lavage samples of the majority of patients with ARDS [34]. Since then, further clinical studies have reported high levels of inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), interleukins (IL)-1β, IL-6 and IL-8 and interferon-gamma (IFN-γ) in the bronchoalveolar lavage (BAL) and/or plasma of patients with ARDS [35-41]. Increasing levels of these cytokines in the BAL and plasma are detected on the first day of ARDS development and the persistence of these markers of inflammation over time is associated with increased mortality [42]. These observations suggest that inflammation is important in both the initiation and progression of ARDS. Intriguingly, only 16% of ARDS patients die due to hypoxia, with most dying from MSOF [43, 44] as a result of inflammation [7, 8].

1.2.4 Lung heterogeneity in ARDS

ARDS is a heterogenous disease [16, 17]. Initially, it was thought that the lung of a patient with ARDS was homogeneous, but Gattinoni *et al.* 1986 [45] reported that there were clearly defined regions of normal (inflatable) and dense (non-inflatable) tissue within a single lung that could be identified by X-Ray Computed Tomography (CT) images. The concept of the "baby lung" was then introduced to describe the small size of the normal (inflatable) region of the lung in ARDS [46, 47]. It is estimated that patients with severe ARDS only have 20% of their lung available for gas exchange [46]. This correlates with patient mortality, with a greater percentage of heterogeneity in non-survivor patients compared with survivors [16]. To date our understanding of the link between lung heterogeneity and the pathogenesis of ARDS is limited.

1.2.5 ARDS treatment

There is no specific treatment for ARDS, rather, the effective management of patients with ARDS involves treating the cause and symptoms [21, 48]. Prolonged ventilation is often required as a lifesaving therapy for patients with ARDS, however, mechanical ventilation itself is thought to worsen the lung condition by inducing injury (i.e. VILI) [49, 50] which contributes to the progression of ARDS, MSOF and mortality [23, 51, 52]. As a result, a high proportion of patients with mild ARDS (55%) show progression of disease to moderate or severe ARDS within a few days of ventilation [24].

VILI occurs through a variety of processes which are likely to be driven by the mechanical response of the lung to positive pressure ventilation. Experimentally, VILI can be induced by ventilating the lung with high tidal volumes (V_T) and/or low positive end expiration pressure (PEEP) [10, 11, 53, 54]. For example, tidal volumes of over 30 mL/kg may be required to induce overstretch injury in experimental models [55, 56]. In line with this, clinical studies have shown that moderate V_T (12 mL/kg) is associated with higher mortality rates than lower V_T (6 mL/kg) [57]. Despite the application of lower tidal volume ventilation strategies clinically, there has been no further decrease in mortality rates over the last decade [3, 33], suggesting that underventilation may also be problematic [58]. However, while the application of PEEP, to prevent lung collapse, was shown to reduce mortality in some studies [20, 59-61], randomised controlled trials have shown no effect of PEEP adjustment on survival rate [62, 63].

There are a number of explanations for this contradiction in observations. For example, the “lung inhomogeneity” hypothesis suggests that the distribution of the stress and strain fields is not uniform which contributes to a heterogeneous response to mechanical ventilation in individual lungs [16, 64]. In addition, the “two-hit”

hypothesis, suggests the outcome is a combination of the nature of pre-existing lung injury and the lung response to the ventilation [18, 65]. Thus, there is both within and between patient heterogeneity that needs to be considered. To date few studies have attempted to determine how individual lung heterogeneity impacts on VILI and how the nature of the pre-existing injury modifies this response.

1.3 Ventilator-induced lung injury (VILI)

1.3.1 The VILI concept

The use of positive pressure ventilation in intensive care units arose in the mid-20th century following the major polio outbreak in Copenhagen in 1953. This clinical intervention was shown to effectively save lives by immediately reducing mortality from 87% to 40% [66]. However, while mechanical ventilation is able to support gas exchange, it does not address the underlying cause of respiratory failure. Paradoxically, positive pressure ventilation is thought to contribute to the high mortality rates in critically ill patients with respiratory failure by inducing lung injury [67]. Mechanical ventilation has been shown to injure the lungs *de novo* [9, 68], which occurs through a range of processes including 1) volutrauma/barotrauma [overdistension with high peak inspiratory pressure (PIP) or high lung volume], 2) atelectrauma (cyclic opening and closing of small airways and recruitment/de-recruitment of the lung units) and 3) biotrauma (inflammation) as a result of either or both baro/volutrauma and atelectrauma [18]. The contribution of these factors to VILI will be discussed in detail below.

1.3.2 Overdistension (barotrauma/volutrauma)

1.3.2.1 Barotrauma

VILI may be caused by barotrauma due to the application of high PIP ventilation [53]. Early clinical studies showed that high PIP ventilation leads to alveolar or pleural rupture resulting in pneumothorax and interstitial emphysema in patients with acute respiratory failure [54]. This was supported by experimental studies. In an early study, Greenfield and colleagues [69] found that healthy dogs ventilated with a high PIP (26-32 cm H_2O) for 2 hours had impaired surfactant function. Meanwhile, Webb and Tierney [68] reported alveolar and perivascular oedema, severe hypoxia and mortality within one hour of ventilation in a group of rats ventilated with high PIP (45 cm H_2O) with no PEEP. Interestingly, the perivascular oedema without alveolar oedema was also found in the groups of rats ventilated with 30 cm H_2O with no PEEP and 45 cm H_2O with 10 cm H_2O PEEP, suggesting that lung injury developed when ventilated with either high PIP or low PEEP [68]. A follow up experiment found that lung oedema was present in groups of rats ventilated with high PIP (45 cm H_2O) for 5, 10 and 20 minutes with the severity of oedema correlating with the duration of ventilation [70]. Histologic examination of lung tissue from these rats found evidence of diffuse alveolar oedema particularly in the alveoli surrounding the broncho-vascular spaces and the subpleural areas. In addition, other studies have shown deterioration of lung function after ventilation at a PIP of 50 cm H_2O in paralysed sheep [55] and increases in microvascular permeability in open chest dogs ventilated with high PIP [71]. In summary, high PIP ventilation causes direct lung injury resulting in oedema and damage to the lung microvasculature.

1.3.2.2 Volutrauma

Volutrauma, as a result of the application of high tidal volumes, is linked to barotrauma, however these processes may also be independent. The concept of volutrauma was proposed by Dreyfuss *et al.* [10], who demonstrated VILI was associated with high tidal volume rather than high-pressure with low V_T ventilation. The high-pressure low V_T condition was achieved by strapping the chest. In that study, oedema was found in the groups of rats, that were ventilated with high V_T, but not necessarily in the groups ventilated with high PIP. The importance of tidal volume was clearly demonstrated by the breakthrough ARDSNet clinical study, which showed a 22% mortality reduction in a group of patients ventilated with 6 mL/kg tidal volume and PIP below 35 cm H_2O compared to a group of patients ventilated with 12 mL/kg [57]. Later experimental studies in animal models showed that high V_T ventilation caused the lungs to deform, which led to development of pulmonary oedema and hyaline membrane formation [56, 72-76]. In some of these studies, PIP was also recorded showing that PIP is inherently higher in the animals ventilated with high V_T [56, 74, 75]. Therefore, volutrauma and barotrauma are not necessarily mutually exclusive. Both can result in overstretch of the alveolar epithelial cells [77] suggesting that it is the process of tissue overdistension that is critical.

1.3.2.3 Overdistension

As describe above, overdistension due to high PIP or high lung volume ventilation has been shown to resulting in vascular permeability, pulmonary oedema and hyaline membrane formation in *in vivo* and *ex vivo* experimental studies. In *in vitro* studies, over-stretch of the alveolar epithelial cells increases cellular permeability, epithelial leakage, and cell death. While stretching of alveolar epithelial type II cells can stimulate surfactant secretion [78], overdistension may affect surfactant-related phospholipid synthesis [79]. Tschumperlin and Margulies [80] hypothesised that

pulmonary alveolar epithelial cells undergo biaxial stretch during mechanical ventilation as the surface area of the basement membrane increases. They found an increase in paracellular permeability and cell death as a result of increasing the stretch magnitude of alveolar epithelial type II cells using a device that generates uniform and equi-biaxial changes in membrane stretch [80, 81]. Cavanaugh *et al.* [82] suggested a strain magnitude threshold exists below which the tight junctions remain intact and exceeding this threshold results in epithelium leakage. Recently, Hamlington and colleagues [83] developed a computational model to study the relationship between stretch force and epithelial leak and found a nonlinear threshold relationship, where the leakage that was initiated after the threshold stretch force had been reached constantly increased as the stretch increased. This finding highlights the importance of overdistension in VILI.

In summary, alveolar overdistension due to high lung pressure/volume ventilation has been shown to have deleterious effects on the lung. On this basis, low lung volume ventilation has been suggested, and used, as a protective strategy [57, 84] to minimise mortality. However, no further survival improvement has been reported since the clinical implementation of the low V_T ventilation [3, 33] suggesting there are other factors that need to be considered.

1.3.3 Cyclic stretch (atelectrauma)

Low lung volume ventilation, primarily due to inadequate PEEP has also been demonstrated to cause VILI through the cyclic opening and closing of small airways (bronchioles) and recruitment/de-recruitment of alveoli [85]; a process referred to as atelectrauma. Initially, Lachmann *et al.* [86] suggested that shear forces, which could be generated by the opening and closing of lung units, can cause damage to the bronchiolar and alveolar epithelium. This mechanism of injury has been noted by

several studies [87-89]. However, those observations were confounded by the application of high inspired oxygen levels, which have also been shown to cause injury to the lung [90]. Muscedere and colleagues [11] used *ex vivo* rat lungs to assess the effect of ventilation at low lung volumes (5 - 6 mL/kg V_T) with different levels of PEEP. A significant decrease in lung compliance and progression of lung injury, with a higher percentage of hyaline membrane formation, was found in the groups ventilated with low or no transpulmonary pressure as compared with the control or higher PEEP groups [11].

Cyclic opening and closing of airways has also been shown to contribute to lung injury resulting in an increase in airway resistance in open-chest rabbits after 3-4 hours of ventilation [91, 92]. A further study in closed chest rabbits showed that ventilation at low lung volumes also increased of lung elastance, which was likely due to interstitial oedema and surfactant depletion or inactivation [93].

The mechanism and effect of the cyclic recruitment and collapse of alveoli has been examined in more recent animal model studies using CT [94-97] and subpleural vital microscopy [98-100]. These studies found that recruitment and de-recruitment also depend on the duration over which low PEEP is applied (breath cycle rate) [101]). These results were supported by a computational study, which suggested that recruitment and de-recruitment is a time dependent phenomenon [102]. In another computational model study, Bilek and colleagues [103] demonstrated that the airway reopening stress cycle, including shear stress, pressure, shear stress gradient and pressure gradient, may inflict significant injury in lung epithelial cells.

In an *in vitro* system, in which the alveolar epithelial cells underwent biaxial stretch, Tschumperlin *et al.* [104] showed that cell death was associated with increasing the

frequency of cyclic stretching. Davidovich *et al.* [105] demonstrated that cyclic stretch induced an increase in alveolar permeability by generating reactive oxygen species (ROS), superoxide, and nitric oxide in Type I rat alveolar epithelial cells. This increase in ROS and superoxide generation has also been demonstrated in immortalized human airway epithelial cell lines in response to cyclic mechanical stretch [106]. On the basis of the studies outlined above, it is likely that atelectrauma makes a significant contribution to VILI.

1.3.4 Lung inhomogeneity

Up to this point, the effects of overdistension and atelectrauma have been considered in isolation. However, the lung has been shown to have a heterogeneous response to ventilation with different stretching profiles (overstretch and atelectasis) occurring in different regions of the same lung [13, 64]. The baby lung concept was initially introduced to explain the fact that different regions of a lung of a patient with ARDS have a heterogeneous response to ventilation [46, 47], where some regions are inflatable while some others are not with this variation depending on disease severity [16]. In the healthy lung, the heterogeneous response of the lung in response to mechanical ventilation has been shown to vary depending on ventilation strategy and gravity [14, 107, 108]. The lung parenchyma is inhomogeneous due to the non-symmetrical distribution of airways, vessels, and the visceral pleura, which leads to regional atelectasis [109]. The stress raiser concept suggests that the stress and strain caused by nonuniform expansion in response to mechanical ventilation, even with moderate pressure and PEEP in a healthy lung, can be harmful [16, 64]. Overall, both baro/volutrauma and atelectrauma may occur concurrently within the same lung.

Since overdistension and atelectrauma lead to similar structural consequences, including increases in microvascular and cellular permeability, a reduction in lung

compliance, hyaline membrane formation and oedema [6], it is unclear how they are developed and which one makes the biggest contribution to VILI.

1.3.5 Biotrauma

Most ARDS patients die from multiple system organ failure (MSOF) [43, 44] which is linked with excessive production of the inflammatory mediators in response to mechanical ventilation [110-113]. Exploration of how VILI related processes (baro/volutrauma and atelectrauma) causes biotrauma, which may contribute to MSOF, is necessary to improve outcomes in patients with ARDS.

1.3.5.1 Biotrauma concept

The term biotrauma was first used by Tremblay and Slutsky [114] to describe the increasing expression of inflammatory mediators in association with mechanical ventilation. Ventilator-induced biotrauma has been demonstrated in in a number of models. *In vivo*, mechanical ventilation leads to neutrophil infiltration and activation [115-117] and increases in platelet activation factor (an important inflammatory mediator) [115]. Using *ex vivo* ventilated rat lungs, Tremblay *et al.* [118] measured lung levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ), chemotactic (macrophage inflammatory protein-2) and anti-inflammatory (IL-10) cytokines, and mRNA levels of *TNF-α* and *c-fos* in response to 2 hours of ventilation with different strategies. Moderate V_T ventilation (15 mL/kg) with 10 cm H_2O PEEP led to increasing levels of TNF-α and mRNA of *TNF-α* and *c-fos*, compared to the control group (7 mL/kg with 3 cm H_2O PEEP). This response increased further when PEEP was reduced to 0 cm H_2O, while the combination of high V_T (45 mL/kg) with no PEEP had a synergistic effect on the production of all measured cytokines [118]. *In vitro* studies using cell lines have confirmed the effect of mechanical stretch on inflammatory cytokine expression whereby 48 hours of 30% mechanical stretch of

alveolar epithelial cells (A549 cells) significantly upregulated the expression of IL-8, even in the absence of structural cell damage [119]. Thus, it is clear, from experimental (*in vivo*, *ex vivo*, and *in vitro* cell line) studies that mechanical ventilation by itself leads to an inflammatory response.

1.3.5.2 Mediators of biotrauma

Since the introduction of this biotrauma concept, the expression of a wide range of genes and proteins, with different biological functions, have been reported in response to mechanical ventilation and/or in ARDS development. These will be summarised in more detail below.

1.3.5.2.1 Surfactant proteins

Pulmonary surfactants are lipoproteins produced by alveolar type II cells. Four surfactant related proteins have been identified and they play a critical role in maintaining lung compliance, facilitating the recruitment of collapsed airway and preventing atelectasis [120]. The levels of surfactant are reduced in respiratory failure [121] and disruption of surfactant protein can cause respiratory failure [122]. In particular, surfactant protein B, a small hydrophobic protein, is reduced in lung injury [123], and treatment by direct application of surfactant protein B can lead to improvement of oxygenation and prevention of the loss of lung compliance [124, 125]. The expression of surfactant protein B has been shown to change in response to mechanical ventilation with different strategies [126], where lower PEEP ventilation was found to be associated with higher levels of this protein compared to higher PEEP. This increase in surfactant production is not consistent with the deleterious effect of low PEEP on lung injury. However, it should be noted that this effect was observed in response to a variable ventilation strategy which is different to conventional ventilation strategies used in the clinic.

1.3.5.2.2 Transcription and cell signalling factors

Many transcription and cell signalling pathways are linked to ARDS development and mechanical ventilation. For example, the expression of c-fos, a transcription marker with a stretch responsive promoter component, is altered in response to mechanical ventilation [114]. Increasing levels of *c-fos* mRNA were found in response to mechanical ventilation, particularly when the lung was ventilated with high V_T and no PEEP [118]. C-fos has a role in regulation of inflammation and cell proliferation and its expression is activated by mitogen-activated protein kinase (Mapk) [127]. The Mapk group, which includes c-Jun, p38, ERK-1 and ERK-2 (Makp-1), responds to various forms of cellular stresses including mechanical ventilation [128] such that high PIP (45 cm H_2O) with 10 cm H_2O PEEP enhances phosphorylation of this group compared to low PIP (13 cm H_2O) and PEEP (3 cm H_2O).

Epidermal growth factor receptor (Egfr), a mechano-transducer, is an upstream regulator of the Mapk response [129]. Egfr is a critical regulator of VILI [130], and is activated by cyclic stretch of lung cells [131, 132]. Nuclear factor kappa B (NF-κB) is another regulator of inflammation and proliferation, that is activated by hypoxia and is found in multiple cell types including alveolar macrophages, lung epithelial cells and pulmonary artery endothelial cells [133]. Levels of this protein increase in response to mechanical ventilation [134]. NF-κB regulates the expression of numerous inflammatory cytokines and chemokines including IL-1β, IL-6, TNF-α, Ccl2, CxCl1 and CxCl2 [134], while nuclear factor erythroid-derived 2-like 2 (Nfe2l2) protects against ARDS development. Nfe2l2 is a transcription factor that coordinates the expression of over 200 antioxidant genes [135]. Degradation of the Nfe2l2 may result in inflammation [136] and Nfe2l2 knockout mice are more sensitive to VILI [137]. Overall, various transcription and cell signalling pathways are involving in the

response to mechanical ventilation and are critical in modulating the inflammatory response.

1.3.5.2.3 Inflammation

A range of inflammatory cytokines and chemokines are associated with the development of ARDS and have been detected in response to mechanical ventilation. For example, TNF-α, IL-1β, IL-6 and IL-8 have been found to increase in patients with ARDS at the onset of disease development and persistence of these cytokines is associated with poor outcomes and mortality [42, 138]. Increases in the expression of these cytokines have also been found in response to mechanical ventilation, especially when high tidal volumes are used, in clinical studies [139, 140] and in *in vivo* [76] and *ex vivo* [118, 141] animal models. In line with this, the increased expression of these cytokines has been demonstrated *in vitro* lung cells in response to mechanical stretch [119, 142]. Upregulation of these inflammatory cytokines is mediated via the NF-κB pathway which also regulates the expression of several chemokines including Ccl2 [134] in response to mechanical ventilation [143, 144]. Ccl2, or Monocyte Chemoattractant Protein-1 (MCP-1), is expressed by mononuclear and epithelial cells and is involved in the recruitment of macrophages to the lung [145-147] in response to mechanical ventilation [148].

Several other inflammatory mediators have also been linked to VILI and ARDS. Receptor for advanced glycation end-products (RAGE) is a multi-ligand receptor that is expressed in alveolar epithelial type 1 cells and is involved in propagation of inflammatory response [149]. Lower levels of RAGE were found in patients on a protective ventilation strategy and there is a strong association between RAGE expression and clinical outcomes in patients with ARDS [150-152]. Angiopoietin-2 (Ang-2) is a predictor of ARDS severity [153, 154], which involved in vascular

endothelial permeability [155]. Ang-2 is associated with pulmonary oedema [153, 154] and lung injury score in mechanically ventilated patients [156]. Neutrophil elastase (Elane) and neutrophil myeloperoxidase (MPO) are potent proteolytic enzymes produced by neutrophils. They are thought to have a role in promoting acute and chronic lung injury [157] and are linked to the tissue damage [158] associated with high tidal volume ventilation [159, 160].

1.3.5.2.4 Blood coagulation

Coagulation is strongly linked to the inflammatory response to injury [161]. Coagulation factors are active in lung tissue in response to pulmonary challenges including mechanical ventilation [162, 163]. Coagulation is involved in the pathophysiology of ARDS through multiple pathways such as tissue factor, protein C and the regulation of fibrinolysis by plasminogen activator (PA) [164]. Tissue type plasminogen activator (Plat), is expressed by endothelial cells and is one of the two PAs that drives the conversion of plasminogen to a fibrinolytic enzyme [164]. A reduction in fibrinolysis is associated with mortality in patients with ARDS [162, 165], while mechanical ventilation with a high tidal volume and no PEEP leads to increasing PA activity [166].

1.3.5.2.5 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a process of epithelial cell proliferation and differentiation in response to injury [167]. EMT is induced by mechanical stretch suggesting it is important in the pathogenesis of VILI [168]. E-cadherin, β-catenin, transforming growth factor β1 (TGF-β1), vimentin and other EMT markers have altered expression in animal models of mechanical ventilation and human epithelial cells exposed to mechanical stretch [169]. E-cadherin, a transmembrane molecule and

epithelial marker of EMT, has a protective role in the formation of epithelial adherent junctions and has its expression decreased in response to high tidal volume ventilation [126, 169-172]. In contrast, the expression of mesenchymal markers of EMT including vimentin, TGF-β and β-catenin increase in response to mechanical ventilation [169]. β-catenin is mediated by wingless integration (Wnt) signalling [173] and can modulate tight junction formation between epithelial cells and protect the alveolar barrier from cyclic stretch [172]. Wnt/β-catenin signalling stimulates tissue remodelling, cell migration, and wound closure. Mechanical ventilation, especially with high V_T, leads to upregulation of Wnt and β-catenin proteins [173].

1.3.6 The impact of baro/volutrauma and atelectrauma on biotrauma

Since the introduction of the biotrauma concept, many clinical and experimental studies have been conducted in order to assess the effect of different ventilation strategies on markers of biotrauma. In a randomized controlled trial, Ranieri and colleagues [139] measured inflammatory mediators (TNF-α, IL-1β, IL-6, and IL-8) in two groups of patients assigned to be ventilated with either conventional (high V_T with low PEEP) or lung-protective (low V_T and high PEEP) ventilation strategies. Thirty six hours after randomisation, the measured BAL levels of inflammatory mediators were significantly increased in the conventionally ventilated group compared to the lung-protective group [139]. Stuber and colleagues [140] demonstrated the effect of different ventilation strategies on the expression of inflammatory cytokines by ventilating 12 mild ARDS patients with a lung-protective strategy (5 mL/kg and 15 cm H_2O PEEP) at baseline then switching them to a conventional ventilation strategy (12 mL/kg and 5 cm H_2O PEEP) for 6 hours before switching back to the lung-protective strategy. The measured plasma levels of IL-1 receptor antagonist, IL-6, and IL-10 were significantly increased at 1 h after switching to conventional ventilation,

and further increased after 6 hours on this strategy, but reduced 1 hour after switching back to the protective strategy and further decreased to baseline levels after 6 hours on the protective strategy. Interestingly, BAL cytokines levels did not decrease after switching from conventional ventilation to a protective strategy [140].

In patients with healthy lungs, Zupancich *et al.* [174] reported that mechanical ventilation led to significant increases in BAL and plasma levels of IL-6 and IL-8 in the group ventilated with 12 mL/kg and 2-3 cm H_2O PEEP compared to those ventilated with 8 mL/kg and 10 cm H_2O PEEP. While, Wrigge *et al.* [175] found only BAL, but not plasma, levels of TNF-α were significantly higher in patients ventilated with high V_T. In contrast, other studies have reported no significant differences in levels of TNF-α, IL-1β, IL-6 and IL-8 in serum of patients with healthy lungs ventilated for 6 hours with different strategies [175-179]. Taken together, clinical studies have shown the varying effects of mechanical ventilation strategies on biotrauma.

In experimental studies, Tremblay *et al.* [180] found levels of TNF-α and IL-6 were ventilator strategy and time dependent. BAL levels of TNF-α and IL-6 mRNA of rat lungs ventilated with 40 mL/kg V_T without PEEP for 30 minutes were significantly increased, whereas those ventilated for 2 hours were the same as the control group. The mRNA levels in the groups ventilated for 2 hours with 15 mL/kg were significantly increased independent of PEEP (either 0 or 10 cm H_2O), suggesting both baro/volutrauma and atelectrauma contributed to the upregulation of TNF-α and IL-6. Another study found that levels of TNF-α, macrophage inflammatory protein-2 (MIP-2), and IL-6 were significantly higher in the group ventilated without PEEP and the highest cytokine levels were found when high V_T and no PEEP were combined [181].

In contrast, Bouadma *et al.* [182] reported that 2 hours of ventilation, even with high V_T (30 mL/kg) and no PEEP, was not sufficient to increase BAL levels of TNF-α, IL-6 and MIP-2 in female BALB/c mice compared with a non-ventilated control group. In a study using a longer duration of ventilation in pigs, Hong *et al.* [183] showed that 8 hours of ventilation with even low V_T (6 mL/kg) and high PEEP (10 cm H_2O) increased the expression of TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12 in the lung. Interestingly, the levels of BAL protein, tissue mRNA, and injury scores in the group of pigs ventilated with high V_T and low PEEP were lower than those ventilated with low V_T and low PEEP suggesting the effect of overstretch and cyclic stretch on biotrauma is complex.

In short, evidence shows that mechanical ventilation leads to upregulation of inflammatory cytokine expression but the magnitude and severity of this response varies considerably depending on ventilation strategy and the relative contribution of baro/volutrauma and atelectrauma to biotrauma remains poorly understood [184]. Cannizzaro *et al.* shed some light on this by showing associations between over-stretch and protein leak, and atelectasis with IL-6 and TNF-α expression [184]. However, as pointed out earlier, it is possible that over-stretch and atelectasis may occur within the same lung to the relative contribution of these to the overall response is unclear.

1.3.7 Biotrauma in the injured lung

The “two-hit” hypothesis suggests that an individual patient’s outcome is a combination of the effect of the existing lung condition that caused respiratory failure and the response of the lungs to mechanical ventilation. This is highlighted by the fact that different results have been found in experimental models with different types of injury. For example, a study by Bouadma *et al.* [182], showed that high V_T (30 mL/kg) without PEEP did not significantly increase levels of TNF-α, IL-6, or MIP-2, but the

combination of injury induced by hemorrhagic shock-resuscitation (HSR) and high V_T led to a significant increase in the levels of these cytokines in the BAL and plasma. Chiumello *et al.* [185] showed that mechanical ventilation after hydrochloric acid instillation led to progressive increases in concentrations of TNF-α and MIP-2 in the lung lavage in rats and that the magnitude of the response increased with high V_T and no PEEP. Interestingly, the rats ventilated with low V_T without PEEP died after 2 to 3 hours of ventilation highlighting the balance between providing adequate ventilation while avoiding lung injury. In a sepsis injury model, Altemeier *et al*. [148] showed that ventilation synergistically enhanced the production of inflammatory cytokines. In contrast, another study showed that mechanical ventilation did not increase the production of TNF-α and IL-6 in female BALB/c mice in response to influenza infection [84]. Therefore, the effect of mechanical ventilation may depend on the nature of the pre-existing lung injury.

1.4 Summary

Mechanical ventilation, a lifesaving strategy for patients with respiratory failure, has been shown to contribute to high mortality rates by injuring the lungs. Baro/volutrauma (overdistension), as a result of high pressure or high tidal volume ventilation, and atelectrauma (cyclic stretch), as a result of low-end expiratory lung volumes, are key mechanical responses that contribute to VILI. Both baro/volutrauma and atelectrauma lead to biotrauma, however, the effect of these on regional biotrauma remains to be determined. When linking the regional variation in the lung's mechanical response to ventilation, and how this relates to biotrauma, it is important to consider how pre-existing lung injury can modify the effect.

1.5 Aims and hypotheses

The overall aim of this study was to assess the impact of mechanical ventilation on regional lung volume and inflammatory responses and how pre-existing lung injury modifies the response. It was hypothesized that the regional inflammatory response to mechanical ventilation depends on the spatial distribution of the local mechanical lung response and pre-existing lung injury.

1.5.1 Specific aims

Aim 1: To assess the association between regional lung volumes and the expression of genes linked to ventilator-induced lung injury in response to different mechanical ventilation strategies in the healthy model.

Hypothesis: The regional lung volume response in the healthy lung to mechanical ventilation depends on the ventilation strategy and is associated with regional gene expression.

Study approach: To achieve this we ventilated two groups of adult female BALB/c mice for 2 hours with either a protective ventilation strategy (moderate PIP with PEEP) or injurious ventilation strategy (high PIP with no PEEP). The regional lung volume responses were assessed by analysing high resolution *in vivo* dynamic 4D CT images taken during ventilation at baseline and after 2 hours of ventilation. The regional gene expression was assessed by measuring mRNA levels of 21 genes in lung tissues collected at the end of the ventilation. Immunohistochemistry was used to confirm the expression of key proteins and to determine regional neutrophilia.

Aim 2: To assess the impact of endotoxemia, as a model of indirect lung injury via systemic inflammation, on the regional lung volume response to mechanical ventilation and how this modifies gene expression.

Hypothesis: The regional lung volume and gene expression response to ventilation with moderate PIP and PEEP will show more extreme regional variation in endotoxemia than in the healthy lung.

Study approach: Endotoxemia in adult female BALB/c mice was achieved by IP injection with LPS in saline or saline alone as a control. Four hours later, the mice were ventilated with a protective ventilation strategy (moderate PIP with PEEP). The regional lung volumes and gene expression responses were assessed by analysing high resolution *in vivo* dynamic 4D CT images and measuring mRNA levels of key inflammatory genes as performed in Aim 1. Immunohistochemistry was used to quantify regional neutrophilia.

Aim 3: To assess the impact of acid aspiration, as a model of direct lung injury, on regional lung volumes and the expression of VILI-related protein in mice in response to mechanical ventilation.

Hypothesis: The regional lung volume and protein expression, response to ventilation, varies regionally in the lung in response to acid aspiration and is fundamentally different to the effect of endotoxemia.

Study approach: Acid aspiration in adult female BALB/c mice was induced by IT instillation of HCl or saline as a control. The mice were then ventilated for 2 hours with a protective ventilation strategy (moderate PIP with PEEP). The regional

lung volumes and protein expression responses were assessed by analysing high resolution *in vivo* dynamic 4D CT images and ELISA respectively.

Chapter 2 - The link between regional tidal stretch and lung injury during mechanical ventilation

Chapter 2 has been removed for copyright or proprietary reasons.

A modified version of this Chapter has been accepted for publication:

Yen, S., Preissner M., Bennett E., Dubsky S., Carnibella R., O'Toole R., Roddam L., Jones H., Dargaville P. A., Fouras A. and Zosky G. R. (**2019**). The Link between Regional Tidal Stretch and Lung Injury during Mechanical Ventilation. American Journal of Respiratory Cell and Molecular Biology **60**(5): p. 569-577. (see Appendix)

This Chapter addresses the first specific aim "To assess the association between regional lung volumes and the expression of genes, linked to ventilator-induced lung injury, in response to mechanical ventilation in the healthy lung." The text of this Chapter is presented as per the published version.

Chapter 3 - The association between regional lung volumes and gene expression during mechanical ventilation with endotoxemia

A modified version of this Chapter has been submitted for publication in *American Journal of Physiology – Lung Cell and Molecular Physiology* (under review):

Yen, S., Preissner M., Bennett E., Dubsky S., Carnibella R., Murrie R., Fouras A., Dargaville P. A. and Zosky G. R. (**Submitted**). The association between regional lung volumes and gene expression during mechanical ventilation with endotoxemia.

In the previous Chapter (Chapter 2), the impact of mechanical ventilation on the regional lung response in healthy lung was assessed. This Chapter aimed to assess the impact of mechanical ventilation on regional lung response in endotoxemia as model of indirect lung injury. The text of this Chapter presented as submitted to the Journal for publication.

3.1 Abstract

Recent studies have suggested that regional variations in inflammation during mechanical ventilation are associated with regional lung overdistension. However, studies in the setting of pre-existing lung injury are limited and have focused on non-specific markers of inflammation. The aim of this study was to assess the impact of endotoxemia on the regional lung volume response to mechanical ventilation and how this modifies regional gene expression.

Adult female BALB/c mice were injected intraperitoneally with 10 mg/kg of *E. coli* derived lipopolysaccharide (LPS) in saline, or saline alone. Four hours later, mice were ventilated for 2 hours with a respiratory rate of 225 breaths/min, a PIP of 12 cm H_2O and a PEEP of 2 cm H_2O. Regional functional residual capacity (FRC) and tidal volume (V_T) were measured at baseline and after 2 hours of ventilation using X-ray velocimetry-based analysis of dynamic high-resolution CT lung images. The regional expression of seven inflammatory genes was quantified by qPCR and regional neutrophilia was quantified on lung sections using immunohistochemistry.

Prior LPS exposure caused region-specific increases in FRC but had no effect on V_T. LPS caused upregulation of all genes measured (*IL-6, Ccl2, TNF-α, CxCl2, IL-1β, MPO, Nfe2l2*). There were regional variations in the expression of *IL-6* ($P = 0.04$) and *Ccl2* ($P < 0.001$) that depended on LPS exposure. In LPS-treated mice *IL-6* expression was negatively correlated with regional FRC whereas, in saline controls, *TNF-α* expression was positively associated with regional FRC. There was no association between the expression of any of the genes measured and V_T.

Our data suggest that pre-existing systemic inflammation modifies the relationship between regional lung volumes and inflammation. In the presence of endotoxemia,

low end-expiratory lung volume promotes the expression of pro-inflammatory pathways.

3.2 Introduction

Mortality rates due to acute respiratory distress syndrome (ARDS) remain high (up to 40%), despite recent advances in clinical interventions [3, 5]. Mechanical ventilation, a lifesaving intervention in ARDS-related respiratory failure, may contribute to the high mortality rate by promoting inflammation in a process known as ventilator-induced lung injury (VILI) [9, 114]. The pathobiology of VILI is complex and is thought to occur through multiple mechanisms including overdistension due to high tidal volume (V_T) or high peak inspiratory pressure (PIP), and atelectrauma due to low positive end expiratory pressure (PEEP) and/or ventilation at low V_T [6].

Optimising ventilation strategies to minimise VILI is complicated by the heterogeneous response of the lungs to mechanical ventilation, whereby overdistension and atelectrauma may occur concurrently in different regions of the same lung [13, 14]. Recently, we found that regional V_T, but not end-expiratory lung volume, is positively associated with the regional expression of *IL-6* and *Ccl-2* suggesting that overdistension is the primary driver of VILI [210]. However, these experiments were conducted in 'healthy' lungs and do not reflect the clinical scenario in ARDS where mechanical ventilation is delivered to a 'pre-injured' lung. Recent studies using positron emission tomography (PET) imaging in sheep have shown that exposure to lipopolysaccharide (LPS) modifies the association between high tidal strain and inflammation (as assessed by ^{18}F-FDG uptake) [191]. However, the nature of this inflammatory response, and whether other pathways are impacted, is unclear.

The two-hit hypothesis suggests that an individual patient's outcome is determined by the interaction between the pre-existing condition which caused respiratory failure and the response of the lungs to mechanical ventilation [18]. For example, endotoxemia

synergistically increases inflammation and cytokine production in response to mechanical ventilation [148]. Similarly, in an experimental model of haemorrhagic shock [182], mechanical ventilation enhanced lung and systemic inflammation while, in a separate study, protective mechanical ventilation had no effect on influenza-induced lung inflammation [84]. Collectively, these observations suggest that prior lung injury has a significant effect on the outcome of mechanical ventilation.

The aim of this study was to assess the impact of mechanical ventilation on regional lung injury in the setting of pre-existing lung injury using a mouse model of endotoxemia. High resolution *in vivo* dynamic four-dimensional computed tomography (4D CT) images were taken during ventilation to quantify regional lung volume responses. These were then correlated with the regional expression of VILI-related genes.

3.3 Methods

3.3.1 Animals

Six- to nine-week-old female BALB/c mice were purchased from the Monash Animal Research Platform (Monash University, Melbourne, Australia). All mice were provided food and water *ad libitum* and housed in a 12:12 hour light-dark cycle. All experiments complied with the guidelines of the National Health and Medical Research Council of Australia and were approved by the Monash University and the University of Tasmania Animal Ethics Committees.

3.3.2 Animal treatment, preparation and ventilation

Mice were injected intraperitoneally with a 200 µL bolus of 0.9% saline (10 mL/kg body weight) with, or without, 10 mg/kg of lipopolysaccharide (LPS) derived from *E. coli O111:B4* (InvivoGen, San Diego, CA, USA). Four hours after injection, mice were anaesthetised (400 mg/kg ketamine, 20 mg/kg xylazine; Troy Laboratories, NSW, Australia), tracheostomised and randomly assigned to either mechanical ventilation or free-breathing groups. Thus, there were four experimental groups (n = 16 per group): 1) saline (control) free-breathing, 2) LPS free-breathing, 3) saline (control) + ventilation and 4) LPS + ventilation. Free-breathing controls were euthanased, after imaging without ventilation, by overdose with sodium pentobarbitone (200 mg/kg) as stipulated by our ethics approval due to the impost on the welfare of the mice in response to systemic LPS administration. The lung tissue was collected and stored in RNA*later*® (Sigma, Australia) for gene expression (n = 8 per group) or fixed in formalin for immunohistochemistry (n = 8 per group). Ventilated mice were prepared as described previously [210] and ventilated with room air (FiO_2 = 0.21) using a small animal ventilator (AccuVent 200, Notting Hill Devices,

Melbourne, Australia) for 2 hours at 225 breaths/minute, with a peak inspiratory pressure (PIP) of 12 cm H_2O, and a positive end-expiratory pressure (PEEP) of 2 cm H_2O. Lung imaging was performed at baseline (H0) and after 2 hours (H2) of ventilation used a custom-built laboratory-based system with a liquid metal jet X-ray source (Excillum AB, Kista, Sweden) coupled with a high-speed detector (PaxScan, Varian Medical Systems, Palo Alto, CA, USA) to capture 4D CT images. This system allowed us to capture high brightness, high resolution images at a high frame rate (at 30 frames per second; 400 projections per CT) [211].

At the end of ventilation, mice were euthanased by sodium pentobarbitone overdose (200 mg/kg) prior to processing of the lung tissue for gene expression (n = 8 per group) or immunohistochemistry (n = 8 per group).

3.3.3 Regional lung volumes

Using offline image processing, a 3D velocimetry technique was applied to calculate regional tidal volume (V_T) [192, 195, 196] while grayscale values (intensity) were converted to Hounsfield Units (HU) to determine the aeration fraction for each voxel to obtain estimates of regional functional residual capacity (FRC). As described previously, the lung was segmented into ten regions (Figure 3.1) with images processed to calculate regional lung volumes [210]. To correct for the variation in regional lung size, we calculated specific FRC (sFRC = regional FRC/regional lung volume), and specific tidal volume (sV_T = regional V_T/regional lung volume) for each region. The 3D velocimetry technique

3.3.4 Regional gene expression

Lungs were removed *en bloc* and tissue was collected from ten regions corresponding to the regional image segmentation (Figure 3.1). Lung tissue was stored separately in

RNAlater® (Sigma, NSW, Australia) at -20 °C prior to processing. RNA extraction on the frozen samples and gene expression assessment using reverse transcription qPCR arrays were performed as previously described [210]. The expression of seven VILI-related genes (*TNF-α*, *IL-1β*, *IL-6*, *Ccl2*, *CxCl2*, *MPO*, and *Nfe2l2*) was assessed, which we have previously shown to vary in response to mechanical ventilation in the healthy lung [210]. Gene expression relative to the reference gene (Rpl37) was calculated using the $2^{-\Delta\Delta CT}$ method [198]. Results were expressed as fold change relative to the average gene expression of a region (L1) in the control free-breathing group.

3.3.5 Immunohistochemistry

In separate groups of mice (n = 8), following euthanasia, lungs were harvested, embedded, sectioned and immunohistochemically stained using anti-neutrophil [NIMP-R14] (5 ng/μL, Abcam, Australia) as described previously [210]. The slides were observed by light microscopy and the number of neutrophils (from at least 20 fields of view) were quantified in randomly selected images within each lung region from a single midline section that captured most of the lung regions. Since R2 and R4 were not consistently present in the midline section, neutrophil numbers were only quantified in eight lung regions.

3.3.6 Data analysis

Differences in sFRC, sV_T, relative gene expression and number of neutrophils, both between regions and between treatment protocols, were assessed using two-way repeated measures ANOVA with Holm-Sidak post hoc tests (SigmaPlot version 12.5, Systat Software Inc. San Jose, USA). Data were transformed using a power transformation where necessary to satisfy the assumptions of normality and homoscedasticity of the variances. Associations between regional sFRC, sV_T and

regional mRNA levels were assessed using linear regression analysis. $P < 0.05$ was considered statistically significant.

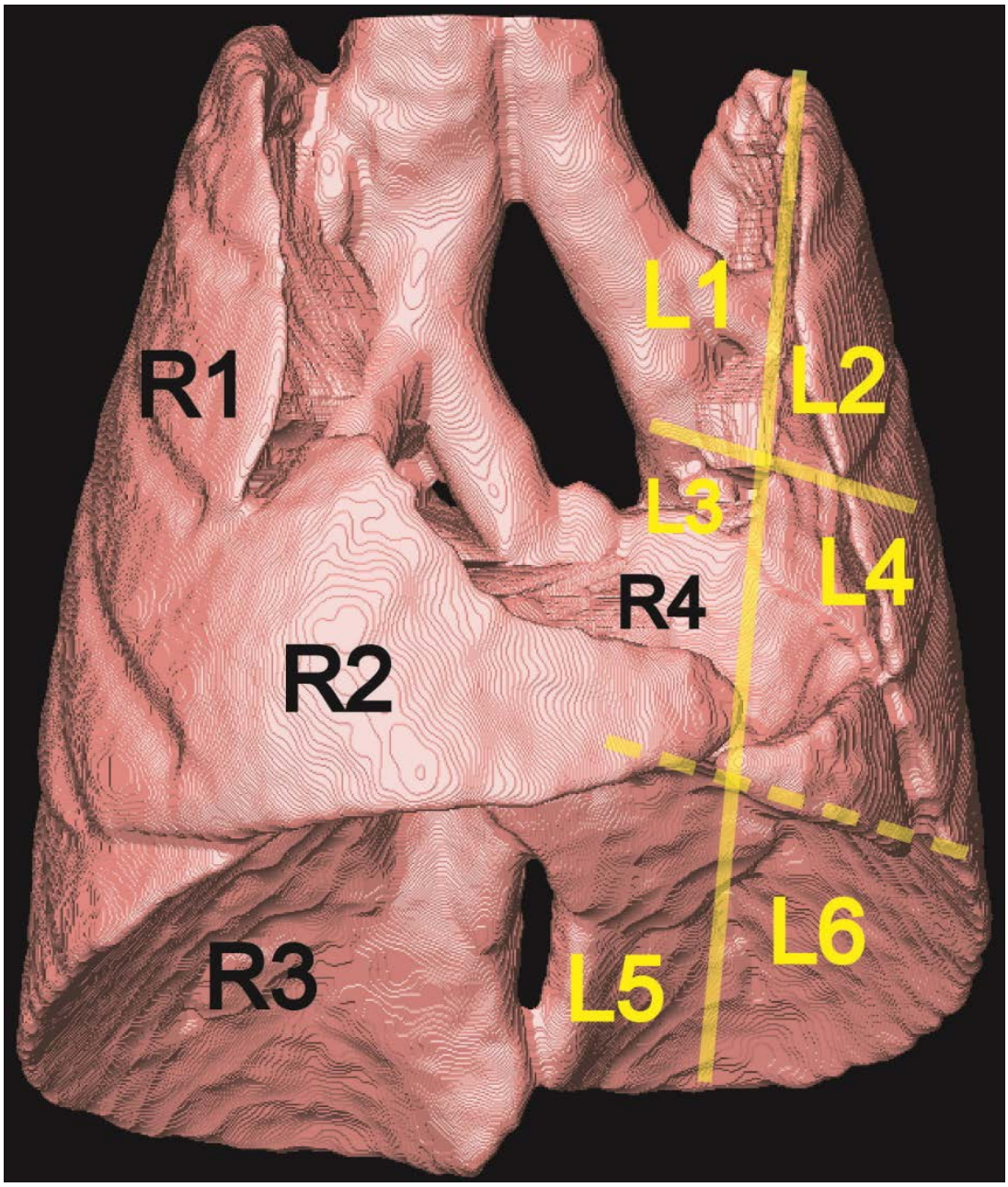


Figure 3.1: Regional lung segmentation. Schematic showing the segmentation approach for the assessment of regional lung volumes. This segmentation corresponded to the tissue sampling process for the assessment of gene expression and image analysis.

3.4 Results

3.4.1 The effect of LPS on lung volumes

Qualitatively, there was significant regional heterogeneity in both FRC (Figure 3.2A and C) and V_T (Figure 3.2B and D). LPS appeared to have minimal impact on V_T, however, there was evidence to suggest that prior LPS exposure increased FRC (Figure 3.2B) in regions of the lung that had low FRC in the controls (Figure 3.2A).

3.4.2 The effect of LPS on sFRC and sV_T at the onset of ventilation (H0)

Regional sFRC varied significantly at H0 and differed between ventilated LPS and control groups ($P < 0.001$) whereby sFRC in L2 ($P < 0.001$) and R1 ($P = 0.01$) were higher in the LPS treated mice (Figure 3.3A). In the control mice, sFRC was higher in the regions proximal to the main conducting airways (L1, $P < 0.001$; L3, $P < 0.001$), the dependent regions at the base of the lung (L5, $P < 0.001$; L6, $P < 0.001$) and the three other right lobes (R1, $P = 0.003$; R2, $P = 0.03$ and R3, $P < 0.001$) (Figure 3.3A) compared to the accessory lobe (R4). In the LPS treated mice all regions had a higher sFRC than R4 at baseline ($P < 0.02$ for all comparisons) (Figure 3.3A). sV_T at H0 varied regionally ($P < 0.001$) within groups, but there was no effect of exposure to LPS on the response ($P = 0.19$) (Figure 3.3B). Within groups, sV_T was higher in the regions proximal to the main conducting airways (L1, $P = 0.002$; L3, $P = 0.001$), the dependent regions at the base of the lung (L5, $P < 0.001$; L6, $P < 0.001$) and the large lower right lobe (R3, $P < 0.001$) (Figure 3.3B).

3.4.3 Change in sFRC and sV_T after 2 hours of ventilation

LPS exposure had no effect on the change in sFRC from H0 to H2 ($P = 0.55$) compared to controls and there were only subtle within-groups regional differences with a significant increase the L5 sFRC relative to R4 ($P = 0.04$) (Figure 3.3C). LPS also had

no effect on the change in sV_T from H0 to H2 (P = 0.12). Within groups, there were regional increases in sV_T that were primarily located in the distal regions of the left lung, L2 (P < 0.001), L4 (P = 0.008), L6 (P = 0.004) and the right upper lobe R1 (P < 0.001) compared to R4 (Figure 3.3D).

3.4.4 Regional gene expression

3.4.4.1 Unventilated mice

Exposure to LPS caused large increases in the expression of all the genes that were measured (P < 0.001 for all comparisons) (Figure 3.4). In saline treated mice, the expression of *Ccl2* was higher in the L3 and R4 regions. mRNA levels were significantly lower in L4 (P = 0.002), L6 (P < 0.001), R2 (P = 0.04) and R3 (P = 0.01) (Figure 3.4C) compared to R4. In contrast, in LPS exposed mice, the expression of *IL-1β* (R3 > L1, P = 0.01) and *MPO* (L2 > R4, P = 0.04) varied regionally (*data not shown*). There were no regional differences in the expression of *IL-6* (P = 0.77; Figure 3.4A), *CxCl2* (P = 0.33; Figure 3.4B), *TNF-α* (P = 0.31 Figure 3.4D) or *Nfe2l2* (P = 0.49; *data not shown*).

3.4.4.2 Ventilated mice

Overall, the regional expressions of all measured genes were significantly higher in the LPS ventilated group than the corresponding regional expressions in the control ventilated group (P < 0.001 for all comparisons). The expression of *IL-6* (P = 0.04) varied regionally within the LPS group, whereby expression in R4 was higher than in several other regions (e.g. vs R1, P < 0.001) (Figure 3.5A). In contrast the expression of *Ccl2* varied regionally but only in the saline treated mice with higher expression in proximal regions of the left lobe (L1, and L3) and lower regions of the right lobes (R3 and R4). Meanwhile, the distal regions (L2, P <0.001; L4 = 0.001; and L6, P = 0.02) of the left lobe had lower expression than the reference region (R4, Figure 3.5C). There

was also regional variation in the expression of *TNF-α* (P = 0.02) and *CxCl2* (P = 0.005), but this was independent of LPS exposure (Figure 3.5B and D). There were no significant differences in regional mRNA levels of *IL-1β* (P = 0.15), *MPO* (P = 0.25) or *Nfe2l2* (P = 0.54) (*data not shown*).

3.4.5 Association between regional gene expression, sFRC and sV_T

In the saline treated mice, regional sFRC was positively associated with *TNF-α* (P = 0.001) expression (Figure 3.6C). In contrast, in the LPS treated mice, the expression of *IL-6* (P < 0.001) was negatively associated with sFRC (Figure 3.6A). There were no further associations between regional lung volumes and the expression of the other genes measured (P > 0.05 for all analysis) (*data not shown*).

3.4.6 Inflammation (neutrophils)

3.4.6.1 Unventilated mice

There was no effect of LPS on neutrophil numbers in the lung tissue (P = 0.23) and there were no regional differences (P = 0.09) in neutrophil density (*data not shown*).

3.4.6.2 Ventilated mice

In ventilated mice, the number of neutrophils varied regionally depending on treatment (P = 0.02, Figure 3.7). LPS exposure significantly increased the number of neutrophils but only in regions L4 (P = 0.02), L5 (P = 0.02), L6 (P = 0.02), R1 (P < 0.001) and R3 (P = 0.007) (Figure 3.7). Unlike the unventilated mice, there was regional variation in both groups such that L1 had higher number of neutrophils than L4 (P = 0.004), L6 (P = 0.007), R1 (P = 0.007) and R3 (P < 0.001) in the saline exposed mice and R1 had higher number of neutrophils than L4 (P = 0.02) in the LPS treated mice (Figure 3.7).

3.4.7 Association between regional inflammation, sFRC and sV_T

There was no association between the number of neutrophils and sFRC and sV_T in either of the groups ($P > 0.3$ for all comparisons; *data not shown*).

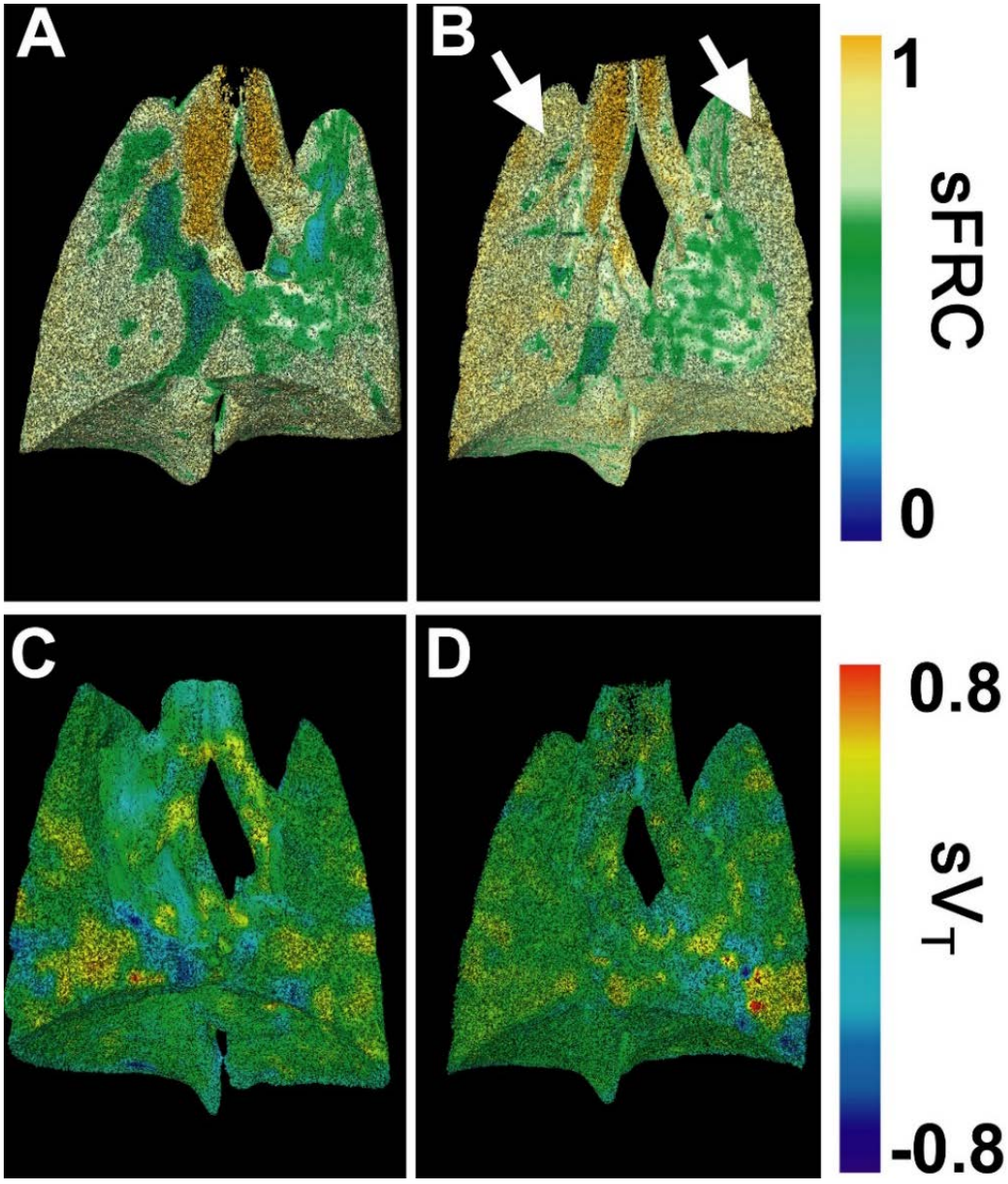


Figure 3.2: Lung imaging during ventilation. Representative 3D images of specific functional residual capacity (sFRC, upper panels) and specific tidal volume (sV_T, lower panels) for a control ventilated mouse (left panels) and an LPS-treated ventilated mouse (right panels). LPS exposure caused an increase in sFRC in regions R1 and L2 (arrows). Regional sV_T was relatively homogeneous at baseline.

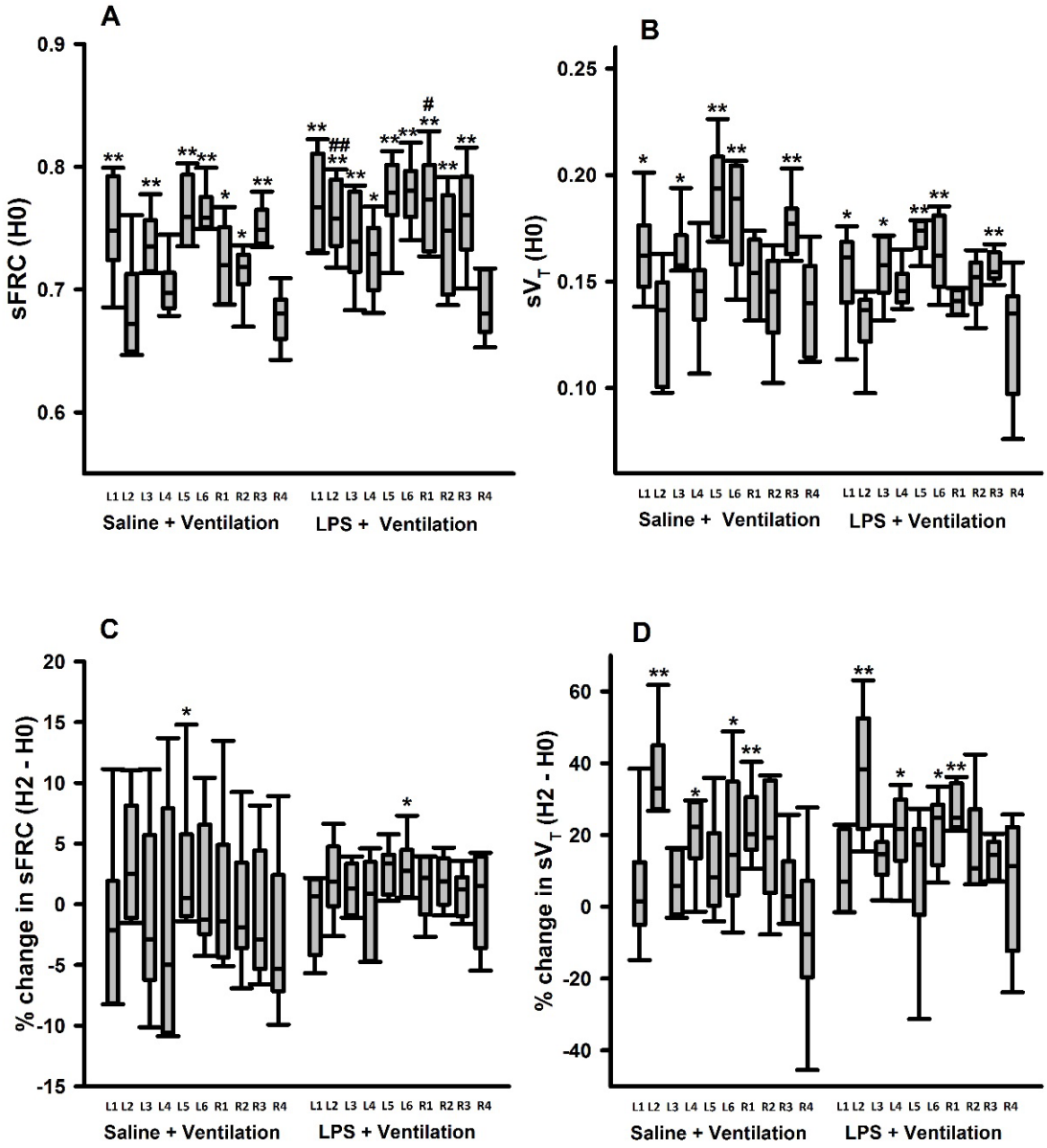


Figure 3.3: Regional lung volumes. Box plots (median, interquartile range, and 10th to 90th percentile) for sFRC and sV_T at baseline (H0; panels A and B respectively) and the proportional change in sFRC and sV_T after 2 hours of ventilation relative to baseline (H2-H0; panels C and D respectively) for each of ten lung regions in control and LPS-treated mice. * and ** indicate $P < 0.05$ and $P < 0.001$ respectively relative to R4 within each group. # and ## indicate $P < 0.05$ and $P < 0.001$ respectively compared with same region in the control group. N = 8 mice per group.

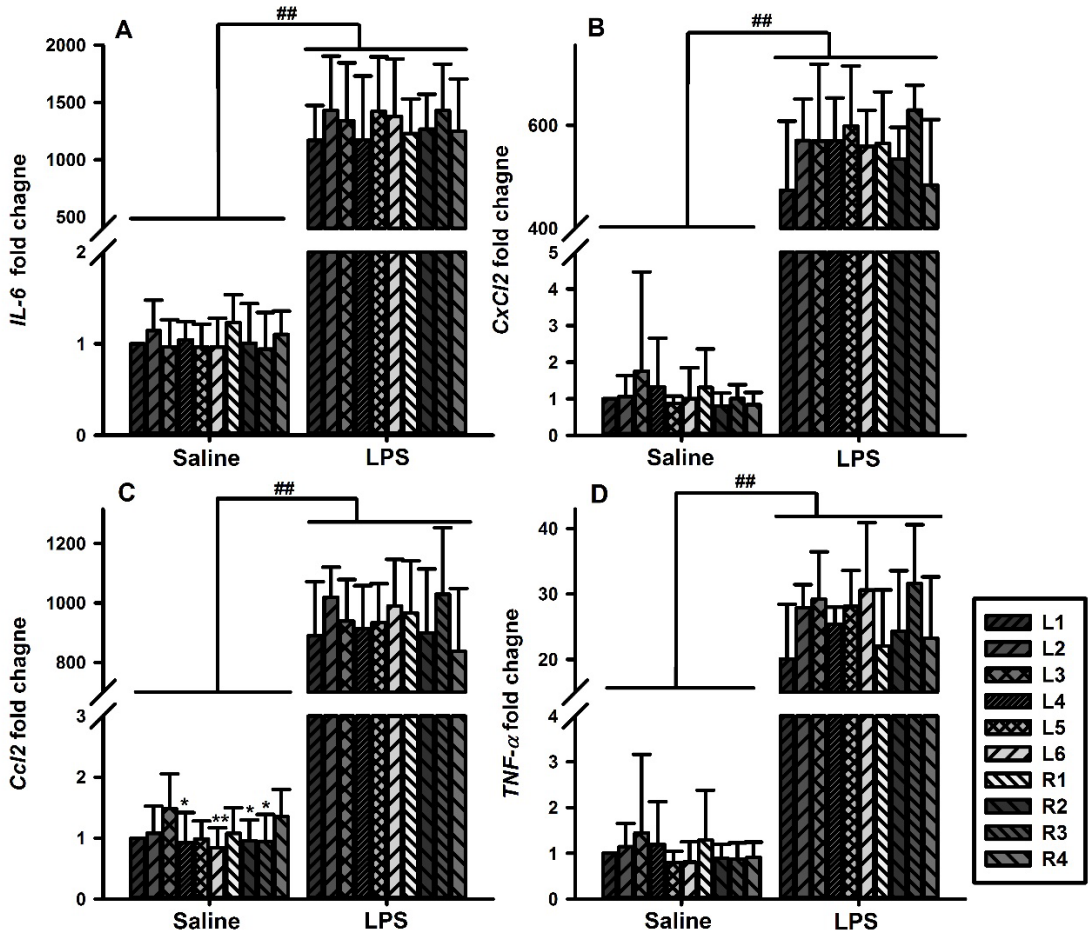


Figure 3.4: Regional gene expression in mice in unventilated mice. Relative fold change of RNA levels was calculated using $2^{-\Delta\Delta CT}$ method relative to the reference gene (*Rlp37*) and average C_T of the L1 region in the control group for *IL-6* (A), *Cxcl2* (B), *Ccl2* (C) and *TNF-α* (D). * and ** indicate $P < 0.05$ and $P < 0.001$ respectively relative to R4 within each group. ## indicates $P < 0.001$ between the two ventilated groups. Data are mean (SD) and N = 8 mice per group.

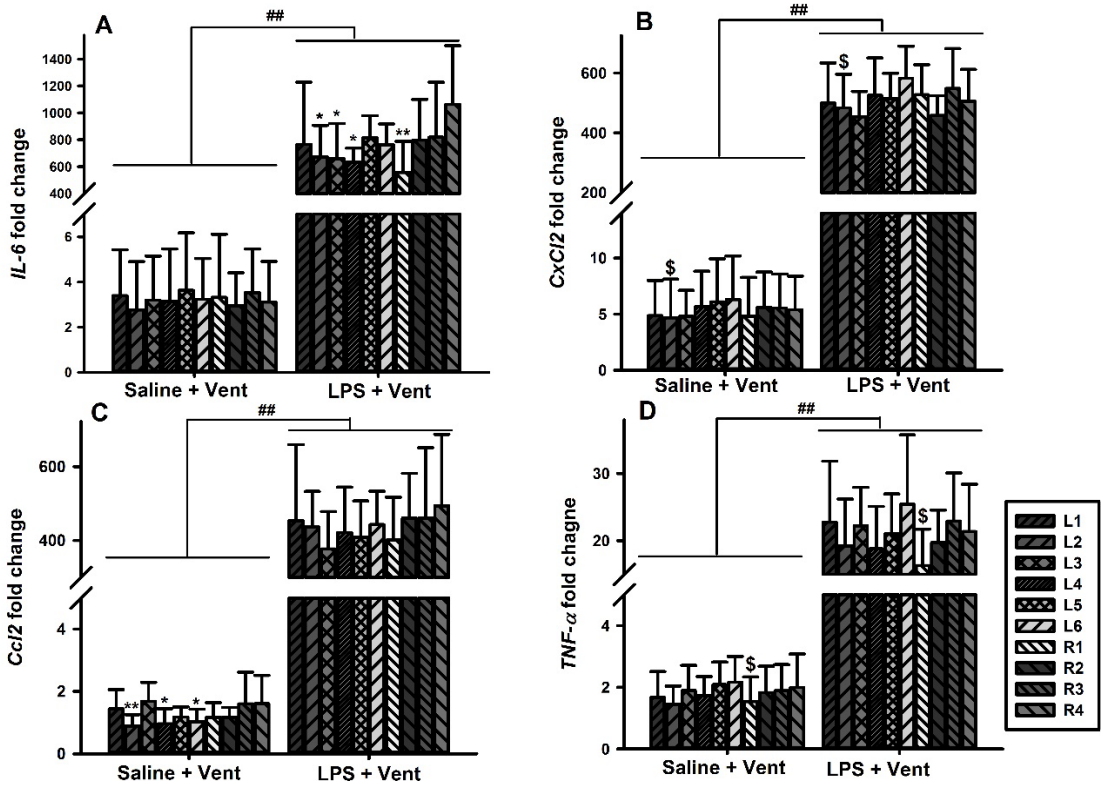


Figure 3.5: Regional gene expression in mechanically ventilated mice. Relative fold change of RNA levels was calculated using $2^{-\Delta\Delta CT}$ method relative to the reference gene (*Rlp37*) and average C_T of the L1 region in the free-breathing control mice for *IL-6* (A), *CxCl2* (B), *Ccl2* (C), and *TNF-α* (D). * indicates $P < 0.05$ relative to R4 within each group. $ indicates $P < 0.05$ relative to L6 within each group. ## indicates $P < 0.001$ between the two ventilated groups. Data are mean (SD) and N = 8 mice per group.

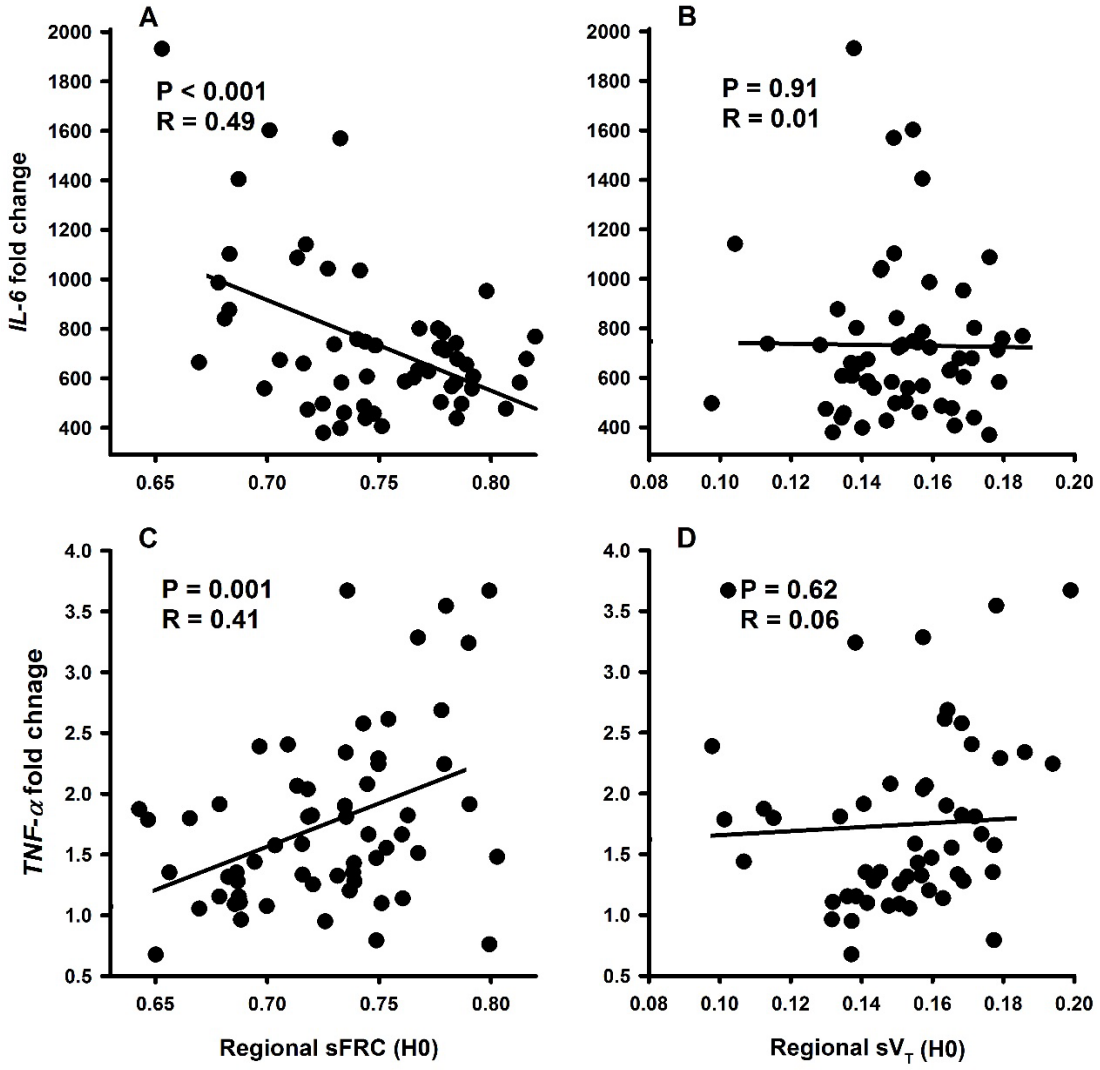


Figure 3.6: Relationship between regional gene expression and lung volumes. Scatterplots showing the relationship between the fold change in regional gene expression of *IL-6*, in the LPS exposed ventilated mice (A, B) and *TNF-α* in the ventilated control mice (C, D), and sFRC (A, C) and sV_T, (B, D). Lines represent the predicted association based on linear regression analysis.

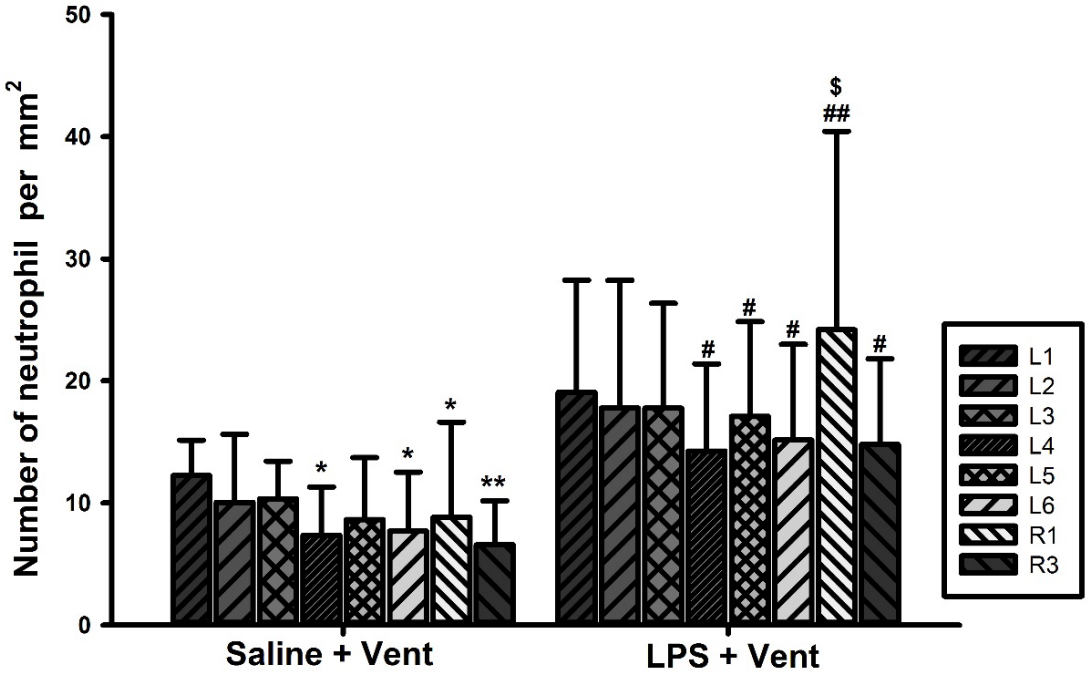


Figure 3.7: Regional numbers of neutrophil in mechanically ventilated mice. Number of neutrophils per square millimetre lung section within each region. * and ** indicate $P < 0.05$ and $P < 0.001$ respectively relative to L1 in the control group. $ indicates $P < 0.05$ relative to L4 within the LPS exposed mice. # and ## indicate $P < 0.05$ and $P < 0.001$ respectively compared with same region in the saline exposed mice. Data are mean (SD) and N = 8 mice per group.

3.5 Discussion

This study aimed to assess the regional response to mechanical ventilation in the setting of pre-existing endotoxemia-induced inflammation. We assessed regional lung volume by analysing high resolution dynamic 4D CT images, taken during ventilation, and examined the association between these volumes and the regional expression of VILI related genes, and inflammatory cells (neutrophils). We found a heterogenous response in regional sFRC whereby pre-treatment with LPS increased gas trapping at end-expiration in some, but not all, lung regions. LPS had no effect on the regional sV_T response. Exposure to LPS increased the expression of all genes measured far above the changes induced by ventilation alone. However, the expression of IL-6 clearly decreased with increasing sFRC suggesting that low lung volume ventilation, in the setting of endotoxemia induced systemic inflammation, increased the inflammatory response. Interestingly, the regional lung volume response did not appear to be associated with the recruitment of neutrophils. Collectively, these data suggest that prior systemic inflammation influences the regional lung response to mechanical ventilation and, in the setting of moderate PIP, low end-tidal lung volumes may promote regional lung injury.

We found regional variations in lung volumes, gene expression and inflammation that were dependent on prior treatment with LPS. While there was little effect on sV_T, there were region specific increases in sFRC in response to LPS, particularly in the regions proximal to the positive pressure being delivered by the ventilator; the upper right lobe (R1) and the point of entry of the main bronchus of the left lobe (L2). This suggests an element of end-expiratory gas trapping. This result is consistent with the observation that the upper region of the lung in ARDS is less likely to collapse [16]. However, a mechanical explanation for this observation, particularly in L2, is unclear. It is possible

that there were areas of dynamic airway collapse, however, in this scenario gas trapping in the distal lung regions would be anticipated, which we did not observe. Related to this, prior lung injury resulting in decreased peripheral compliance could cause a redistribution of regional tidal volumes such that the central lung regions become more compliant as a result of the additional tidal stretch [195, 212]. Alternatively, it is possible that this increase in sFRC represents local barotrauma at the pressure front of the ventilation waveform [103, 213, 214], however, we saw no evidence of overt lung injury in these regions in the LPS treated mice and this is not consistent with the gene response we observed.

In the LPS treated mice there was a significant negative correlation between *IL-6* and sFRC but not sV_T suggesting that regional low end-expiratory lung volume is, potentially, detrimental in the setting of systemic inflammation. The expression of IL-6, an inflammatory cytokine, is associated with poor outcomes in ARDS [37, 138] and elevated in response to overdistension and cyclic stretch of lung tissue [118, 140]. The stretch related IL-6 response has been shown to be suppressed by the application of moderate PEEP [215, 216], but is enhanced by high PEEP [215] due to overdistension [139]. In line with this, we have previously shown that the expression of *IL-6* in the healthy lung is positively associated with sV_T [210]. However, the data in the present study are inconsistent with this observation. There are two things worth noting when considering this discrepancy. Firstly, in our previous study, we used a range of ventilator settings which included much higher inspiratory pressures (up to 20 cm H_2O). Thus, it is possible that the position association we previously observed between *IL-6* expression and sV_T only manifests at high PIP and avoidance of such ventilation strategies, as shown in the ARDSNet trial [57], is beneficial. Secondly, there is a fundamental experimental difference in the current study; pre-existing systemic injury. This could have multiple effects on the response. As discussed earlier, prior injury is

likely to decrease peripheral compliance which will promote atelectasis and cyclic recruitment and de-recruitment of lung units; a phenomenon that has been shown to promote lung injury [6, 11]. In addition, prior exposure to LPS significantly altered the inflammatory milieu, as shown in the mRNA data, which is likely to fundamentally change the tissue response to stretch [217, 218]. While a clear explanation for this observation is not possible based on our data, we have demonstrated that prior systemic inflammation fundamentally alters the tissue stretch response in the setting of moderate PIP and PEEP. In particular, low regional lung volume ventilation, as indicated by low sFRC, appears to promote an inflammatory response in the lung.

In support of the notion that prior injury alters the response, in the uninjured lung, we found a positive correlation between *TNF-α* and regional sFRC. TNF-α is a pro-inflammatory cytokine which has been shown to increase in expression in response to LPS [219] and mechanical ventilation [203]. While some studies have shown that mechanical ventilation can enhance the production of TNF-α in the previously injured lung [148, 220, 221], others have found ventilation alone has no effect on TNF-α production [118, 222]. Our data suggest that the effect of mechanical ventilation on TNF-α production is related to regional overdistension (sV_T), but only in the "healthy" lung. Again, this highlights the effect of prior injury on the relationship between tissue stretch and the inflammatory response induced by mechanical ventilation.

To further assess the impact of ventilation on inflammation, we also quantified regional neutrophil levels. Neutrophils, a critical inflammatory cell in ARDS pathogenesis [34], have been shown to increase in response to both LPS treatment and mechanical ventilation [223, 224]. In the unventilated mice, there was no effect of LPS treatment on the number of neutrophils in the lung, however, in the ventilated mice, prior LPS treatment significantly increased neutrophil numbers in a region dependent

manner. Interestingly, this regional variation in neutrophil numbers was not associated with our measures of tidal stretch or end-tidal lung volume. This is surprising given the strong association between IL-6 production and neutrophil infiltration [225-227] suggesting a disconnect between the cytokine response and the recruitment of neutrophils. It is possible that this is due the short duration of ventilation such that the recruitment of neutrophils had not peaked after 2 hours of ventilation. Future studies may consider longer ventilation protocols to fully characterise the kinetics of the inflammatory response with a focus on regional heterogeneity.

This study had some limitations which should be acknowledged. Firstly, the ventilation protocol was limited to a single PIP and PEEP strategy. Ventilation with different strategies, particularly with adjustment of PEEP, given the effect of low FRC we have shown, may give greater insight into the impact of ventilation on regional lung injury in the previously injured lung. Also, in order to fulfil animal welfare requirements free-breathing mice were euthanased 4 hours after exposure to LPS which limited our capacity to dissect the effect of ventilation alone on the inflammatory response. Nonetheless, within the ventilated animals, we were able to clearly demonstrate important associations between regional lung volumes and the gene response and how this is influenced by prior systemic inflammation.

In conclusion, we have demonstrated that regional variation in sFRC is altered by endotoxemia. Interestingly, while we have previously shown that overstretch is a key driver of regional inflammation in the healthy lung, we found that the endotoxemic lung is susceptible to low lung volume ventilation. Further investigation of the impact of different ventilation strategies and lung injuries on the regional response may provide further insight into the pathogenesis of ARDS in response to mechanical ventilation.

Chapter 4 - Regional inflammatory cytokine expression and lung volumes in response to mechanical ventilation in the acid aspirated lung

In the previous Chapters, the impact of mechanical ventilation on the regional lung response was assessed in the healthy lung (Chapter 2) and with endotoxemia as a model of indirect injury (Chapter 3). This Chapter aimed to assess how the regional lung response to mechanical ventilation in acid aspiration induced direct lung injury and whether the response is different to what was observed with indirect injury (Chapter 3). The text of this Chapter is presented as a “traditional” Thesis Chapter (i.e. has not been formatted for publication).

4.1 Abstract

In the previous Chapters the effect of mechanical ventilation on regional lung stretch and lung injury in the healthy (Chapter 2) and endotoxemic (Chapter 3) lung was assessed. However, it is unclear whether these observations are altered in the setting of different lung injuries that can cause ARDS. This study aimed to assess the impact of ventilation on regional lung volumes and VILI-related protein expression in mice exposed to acid aspiration as a model of direct lung injury. It was hypothesized that the lung response to ventilation would vary regionally depending on exposure to acid and that the response would be fundamentally different to that observed in Chapter 3 using a model of indirect injury.

Two groups of adult female BALB/c mice were randomly assigned to receive intratracheally administration of 50 µL of HCl (pH = 3) or 0.9% saline. Groups of mice were further divided into free-breathing controls, which were euthanased 30 minutes after acid administration, or mechanically ventilated for 2 hours with a respiratory rate of 225 breaths/min, a PIP of 12 cm H_2O and a PEEP of 2 cm H_2O. Regional lung volumes were obtained from analysis (x-ray velocimetry) of dynamic high-resolution 4D CT lung images at baseline and after 2 hours of ventilation to calculate regional specific tidal volume (sV_T) and specific functional residual capacity (sFRC). Ventilated mice were euthanased at the end of the ventilation period. Lungs were harvested from all mice, and divided into ten regions, for assessment of regional expression of four inflammatory proteins (TNF-α, MCP-1, IL-1β and IL-6) by ELISA.

There were variations in regional sFRC ($P < 0.001$) and sV_T ($P < 0.001$), however, exposure to acid aspiration had no effect on sFRC ($P = 0.15$) or sV_T ($P = 0.88$). There were no changes in regional lung volumes from baseline to the end of the ventilation

period. While there were regional differences in protein expression, HCl exposure had no effect on the expression ($P > 0.67$ for all proteins). Similarly, ventilation had no effect on the level of the measured proteins ($P > 0.23$ for all comparisons) and there was no association between regional lung volumes and protein expression ($P > 0.17$ for all comparisons). In order to explore the effect of IT fluid administration on the response, lung volume data from the control group in Chapter 3 (IP saline exposed) was compared with data from the control group in this Chapter (IT saline exposed). IT exposure to saline significantly increased sFRC in some regions ($P = 0.01$) and global sV_T ($P = 0.04$).

In conclusion, there was no effect of acid aspiration or ventilation on the regional lung volume response. This is likely due to the combined effect of the pH that was too high to induce significant injury and the effect of IT fluid administration on the stretch response to mechanical ventilation. On the basis of these data, it is unclear whether direct lung injury alters the regional stretch and inflammatory response compared to indirect lung injury.

4.2 Introduction

In the previous Chapters, the impact of mechanical ventilation on regional lung injury in the healthy lung (Chapter 2) and in the pre-injured lung (Chapter 3), using a model of endotoxemia, were assessed. In the healthy lung, there were variations in regional lung volumes (sFRC and sV_T) and the expression of inflammatory genes in response to mechanical ventilation [210]. The expression of two genes, *IL-6* and *Ccl2*, varied regionally depending on the ventilation strategy. The link between regional lung volumes and gene expression was demonstrated by a positive association between the regional expression of *Ang-2, IL-6,* and *Ccl2* and regional sV_T, but not sFRC, suggesting that regional over-inflation has the greatest impact on injury in the healthy lung.

Consistent with the healthy lung, there was also regional variation in the expression of *IL-6* and *Ccl2* in the setting of pre-existing inflammation (endotoxemia) (Chapter 3). However, the expression of these genes was not associated with sV_T. In contrast, in the LPS treated mice, there was a negative association between sFRC and the expression of *IL-6* suggesting that low lung volume ventilation is the most detrimental in the pre-injured lung. These findings show that regional ventilator-induced lung injury is dependent on ventilation strategy and the pre-existing lung condition. However, it is unclear whether this observation is true for all types of prior lung injury that can contribute to the development of ARDS.

ARDS is caused by various clinical disorders which can be broadly classified into direct and indirect injuries to the lung. Sepsis is the major cause of ARDS development as a result of indirect injury to the lung [19, 21, 23, 24]. On the other hand, aspiration, the inhalation of foreign materials such as food particles, liquids, blood and bacteria,

into the airways [228, 229], is the common causes of direct lung injury [21, 27, 28]. Patients with suspected aspiration are more likely to develop a severe form of ARDS leading to a high mortality rate [23, 24]. The inhalation of low pH regurgitated gastric contents may cause chemical injury to the airways and parenchyma leading to the activation of neutrophils and the development of ARDS [228, 230, 231]. Gastric aspiration is the common cause of aspiration and frequently occurs in ICU or trauma patients, where there is an altered state of consciousness [228]. This situation arises in one in every 2-3 thousand of patients undergoing anaesthesia [228, 232, 233] and up to 20% of deaths linked to anaesthesia are thought to be due to gastric aspiration [228, 234].

In order to study the effects of gastric aspiration on the lung, a number of models of gastric aspiration have been established, typically by oropharyngeal instillation of a low pH liquid. The effect of acid aspiration on the lung is depended on pH and fluid volume [230]. The severity of the lung injury increases when the acidity or volume of fluid delivered increases. Experimental models of the impact of acid aspiration on the lung have shown that the response is characterised by deterioration of gas exchange, development of oedema, alterations in surfactant function, loss of lung compliance, increasing alveolar permeability, inflammation and the generation of reactive oxygen species [230, 235-245]. Mechanical ventilation and exposure to acid aspiration prior to, or during, ventilation has been shown to cause lung injury as demonstrated by increased airways hyperresponsiveness, microvascular permeability, enhanced expression of IL-8 and neutrophil recruitment [246-248]. The impact of ventilation and acid aspiration is also dependent on ventilation strategy. Injurious ventilation with high PIP or V_T and no PEEP induces more severe injury, while protective ventilation with moderate PIP and PEEP induces minor impact [185, 249], suggesting that variations in lung volume may alter the response.

Little is known about the impact of ventilation on the regional lung response in aspiration. A recent study assessing the regional inflammatory response in mechanically ventilated rat using hyperpolarized (HP) carbon-13 magnetic resonance imaging (MRI) [250] found increases in the regional expression of broad inflammatory markers in the acid aspirated rats ventilated with no PEEP. However, the analysis was only based on 2-D slices of the images and the range of inflammatory markers measured was limited.

The aim of this Chapter was to assess the impact of ventilation on regional inflammation in a model of pre-existing, direct, lung injury by ventilating mice after intratracheal administering hydrochloric acid. It was hypothesized that the lung response to ventilation would vary regionally depending on exposure to acid aspiration and that the response would be fundamentally different to that observed in Chapter 3 using a model of indirect injury. The impact of the acid aspiration and ventilation on lung volumes at the regional level was assessed by analysing high resolution *in vivo* dynamic 4D CT images, taken during ventilation as per the previous Chapters. In the previous Chapters, the expression of inflammatory genes was assessed by measuring their mRNA levels. However, this has limitations as the expression levels may not translate into altered protein expression. Therefore, in this Chapter, regional protein levels were assessed.

4.3 Methods

4.3.1 Animals

Six- to nine-week-old female BALB/c mice were purchased from the Monash Animal Research Platform, Monash University (Melbourne, Australia). All mice were provided food and water *ad libitum* and housed in a 12:12 hours light-dark cycle. All experiments complied with the guidelines of the National Health and Medical Research Council of Australia for the Care and Use of Animals for Scientific Purposes (2004) and were approved by the Monash University and University of Tasmania Animal Ethics Committees.

4.3.2 Animal preparation and ventilation

The animals were prepared as described in Chapter 2. Briefly, they were anaesthetized with intraperitoneal (IP) injection of a solution containing 400 mg/kg ketamine (Troy Laboratories, NSW, Australia) and 20 mg/kg xylazine (Troy Laboratories, NSW, Australia). Initially, two-thirds of the dose was given to induce a surgical plane of anaesthesia before tracheostomy and insertion of a 10 mm polyethylene cannula (0.86-mm inner diameter). Top-up doses of the anaesthetic were given throughout the ventilation period when necessary. Following tracheostomy, the animals were placed in a customised 3D-printed holder and randomly selected for treatment by intratracheal administered with either 50 μL of 0.9% saline or hydrochloric acid (HCl, pH = 3, Sigma-Aldrich, Castle Hill, NSW, Australia). As stipulated by our ethics approval, due to the impact on the welfare of the animals, the free-breathing controls mice were euthanased 30 minutes after HCl administration by overdose with sodium pentobarbitone (200 mg/kg).

Ventilated mice were mounted on a rotating stage (Zaber Technologies, Vancouver, Canada) in a custom-built holder. The tracheal cannula was attached to a small animal ventilator (AccuVent 200, Notting Hill Devices, Melbourne, Australia) operating in a pressure-limited mode. Mice were ventilated with room air (FiO_2 = 0.21) for 2 hours at 225 breaths/min with a PIP of 12 cm H_2O and 2 cm H_2O PEEP. Lung images of the ventilated groups were taken at baseline (H0) and after 2 hours (H2) of ventilation. Mice were euthanased at the end of ventilation by overdose with sodium pentobarbitone (200 mg/kg) prior to processing of the lung tissue for protein expression (n = 8 per group).

4.3.3 X-ray imaging

The X-ray imaging setup was as described in Chapters 2 and 3.

4.3.4 Post-processing of imaging data

Images were processed for quantification of regional specific FRC (sFRC) and specific V_T (sV_T) for ten lung regions as described in Chapters 2 and 3.

4.3.5 Protein expression

Following euthanasia, the lungs were removed *en bloc* and divided into 10 regions consisting of the four right (R1, R2, R3 and R4) lobes and six regions (L1, L2, L3, L4, L5 and L6) from the left lobe corresponding to the image analysis (Figure 3.1). Each lung region was snap frozen in liquid nitrogen before being stored at - 80 °C prior to analysis.

Protein extraction was performed using T-PER® Tissue Protein Extraction (Thermo Scientific, Rockford, USA). Briefly, the lung tissues were homogenized in T-PER

reagent (20 µL/g tissue) using a T10 basic ULTRA-TURRAX homogenizer system (IKA, Wilmington, USA). The mixture was centrifuged at 10,000 *g* for 5 minutes to pellet cell/tissue debris and supernatant was collected and stored at - 80 °C. Total protein was quantified using a Coomassie (Bradford) protein assay kit (Thermo Scientific, Rockford, USA) by according to the manufacturer's instructions.

The level of TNF-α, MCP-1, IL-1β and IL-6 in tissue homogenate was measured using Mouse DuoSet ELISAs (R&D System, Minneapolis, USA) according to the manufacturer's instructions. The absorbance was read at 450 nm or 570 nm with spectrophotometer (Spectramax M2; Molecular Devices, Sunnyvale, CA, USA). The relative regional protein levels were normalised to the total protein.

4.3.6 Data analysis

Two-way repeated measures ANOVA with Holm-Sidak *post hoc* tests (SigmaPlot version 12.5, Systat Software Inc. San Jose, USA) were employed to assess regional differences in sFRC and sV_T and relative protein expression, both between regions and between treatments. Data were transformed using a power transformation where necessary to satisfy the assumptions of normality and homoscedasticity of the variances. Associations between regional sFRC, sV_T, distension and regional protein levels were assessed using linear regression analysis. $P < 0.05$ was considered statistically significant and data are expressed as mean (SD).

4.4 Results

4.4.1 Lung volume (sFRC and sV_T) at baseline

At baseline (H0), regional sFRC varied ($P < 0.001$), independent of treatment ($P = 0.15$), (Figure 4.1A), whereby the accessory lobe (R4) had significantly lower sFRC than all other regions in both the acid aspiration and saline groups ($P < 0.001$ for all comparisons). In addition, the regions distal to the main conducting airways, such as L2, L4 and R2, had lower sFRC than the other six regions ($P < 0.05$ for all comparisons) (Figure 4.1A). Similarly, sV_T at H0 varied regionally ($P < 0.001$), but there was no effect of treatment on the response ($P = 0.88$) (Figure 4.1B). sV_T in R4 was significantly lower than all other regions in both groups ($P < 0.001$ for all comparisons) (Figure 4.1B).

4.4.2 Change in sFRC, sV_T and regional distension after 2 hours of ventilation

Overall there was no significant change in sFRC ($P = 0.31$) or sV_T ($P = 0.42$) from baseline after 2 hours of ventilation (H2 vs H0). While there was considerable variation between animals, exposure to acid had no effect on the response (sFRC, $P = 0.45$; sV_T, $P = 0.53$) (Figure 4.1C and D).

4.4.3 Regional protein expression

4.4.3.1 Free-breathing mice

Exposure to HCl had no effect on the level of any of the measured proteins in the unventilated mice ($P > 0.67$ for all comparisons) (Figure 4.2). There were, however, variations in regional protein levels within groups. With L1 as the reference, the level of IL-6 was significantly lower in R1 ($P = 0.048$) (Figure 4.2A), while the levels of IL-1β were lower in R1 ($P = 0.003$), R2 ($P = 0.006$) and R3 ($P = 0.001$) (Figure 4.2B)

and TNF-α expression was lower in L4 ($P = 0.004$), R1 ($P < 0.001$), R2 ($P < 0.001$) and R3 ($P < 0.001$) (Figure 4.2D). There was no regional difference in the expression of MCP-1 ($P = 0.08$) (Figure 4.2C).

4.4.3.2 The effect of ventilation (within saline treated groups)

Mechanical ventilation had no effect on the expression of any of the measured proteins in the saline treated mice ($P > 0.12$ for all comparisons) (Figure 4.3).

4.4.3.3 The effect of ventilation and acid aspiration

Exposure to HCl had no effect on the level of any of the measured proteins in the ventilated mice ($P > 0.23$ for all comparisons) (Figure 4.4). However, there were significant regional variations in protein expression levels in the ventilated mice with the two regions proximal to the main conducting airways (L1 and L3) having higher levels of all measured proteins compared to the other eight lung regions ($P < 0.05$) (Figure 4.4).

4.4.4 Association between regional gene expression, sFRC and sV_T

The linear regression analysis showed no significant association between regional lung volume (sFRC or sV_T) and IL-1β, IL-6, MCP-1 or TNF-α levels ($P > 0.17$ for all comparisons) (Figure 4.5).

4.4.5 Effect saline treatment (IP and Aspiration) on lung structure

Because there is no significant effect of acid aspiration on lung volume response and the effect of fluid instillation is concerned, the effect of saline aspiration on the lung volume response was assessed by compared with the response after an IP injection of saline (Chapter 3). The impact of mechanical ventilation on the lung volume response varied depending on method of administration of saline, whereby the regional sFRC at H0 was dependent on the mode of saline treatment ($P = 0.01$). In particular, the

sFRC in L2 (P = 0.04) and R1 (P = 0.03) was higher in the aspirated group than the IP injected group (Figure 4.6A). In addition, the saline aspirated group had higher global tidal volume than the IP injected group (P = 0.04) (Figure 4.6B).

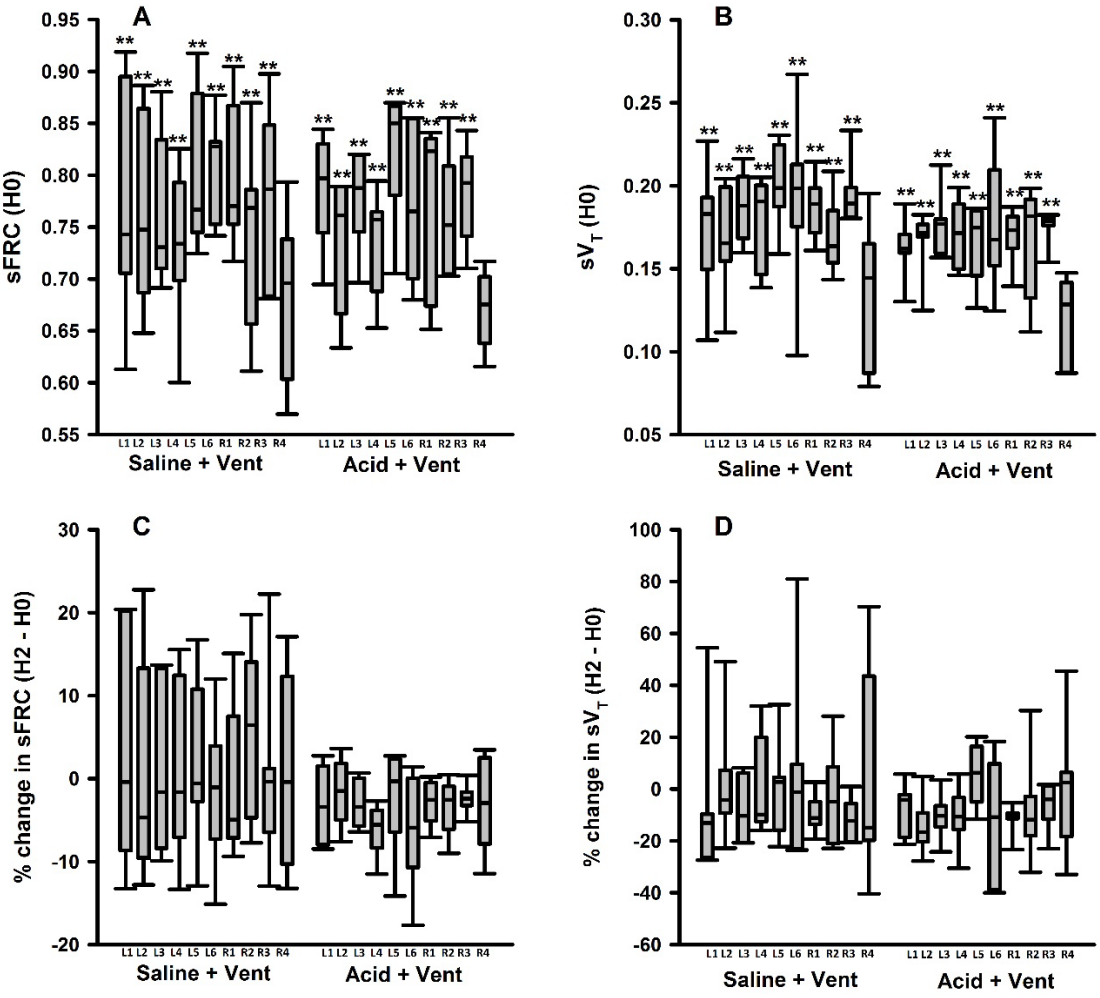


Figure 4.1: Effect of acid aspiration on lung volumes in ventilated (Vent) mice. Box plots (median, interquartile range, and 10th to 90th percentile) for sFRC and sV_T at baseline (H0; panels A and B respectively) and the proportional change in sFRC and sV_T (panels C and D respectively) after 2 hours of ventilation relative to baseline for each of ten lung regions in mice from each ventilated group. * and ** indicate P < 0.05 and P < 0.001 respectively relative to R4 within each group. N = 8 mice per group.

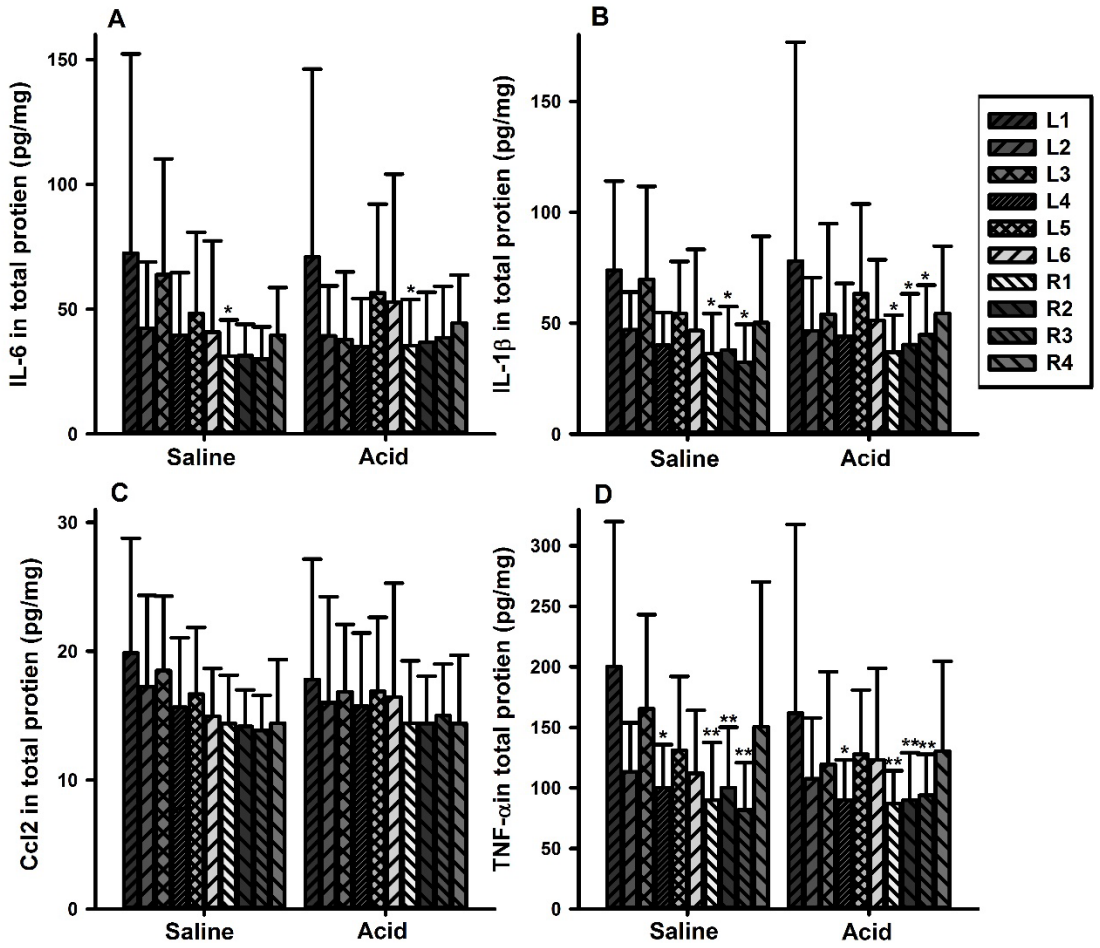


Figure 4.2: Regional protein levels in free-breathing mice. Levels of the proteins IL-6 (A), IL-1β (B), Ccl2 (C), and TNF-α (D) in the saline controls and acid treated free-breathing mice were measured by ELISA. The levels of the proteins are presented as a proportion of total protein, which was measured by Bradford assay. * and ** indicate $P < 0.05$ and $P < 0.001$ respectively relative to L1 within each group. N = 8 mice per group.

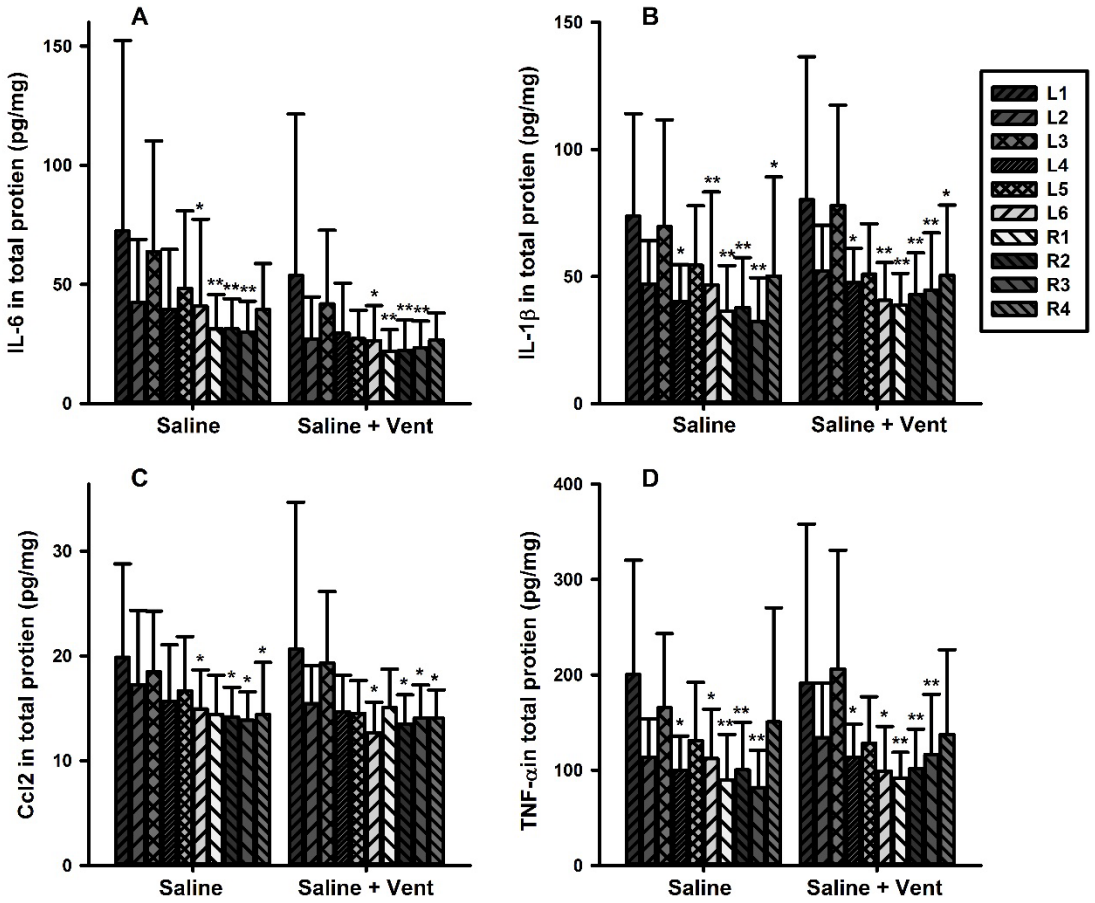


Figure 4.3: Regional protein levels in mechanically ventilated and free-breathing saline controls. Levels of the proteins IL-6 (A), IL-1β (B), Ccl2 (C), and TNF-α (D) in each lung region were measured by ELISA. The levels of the proteins are presented as a proportion of total protein, which was measured by Bradford assay. * and ** indicate $P < 0.05$ and $P < 0.001$ respectively relative to L1 within each group. N = 8 mice per group.

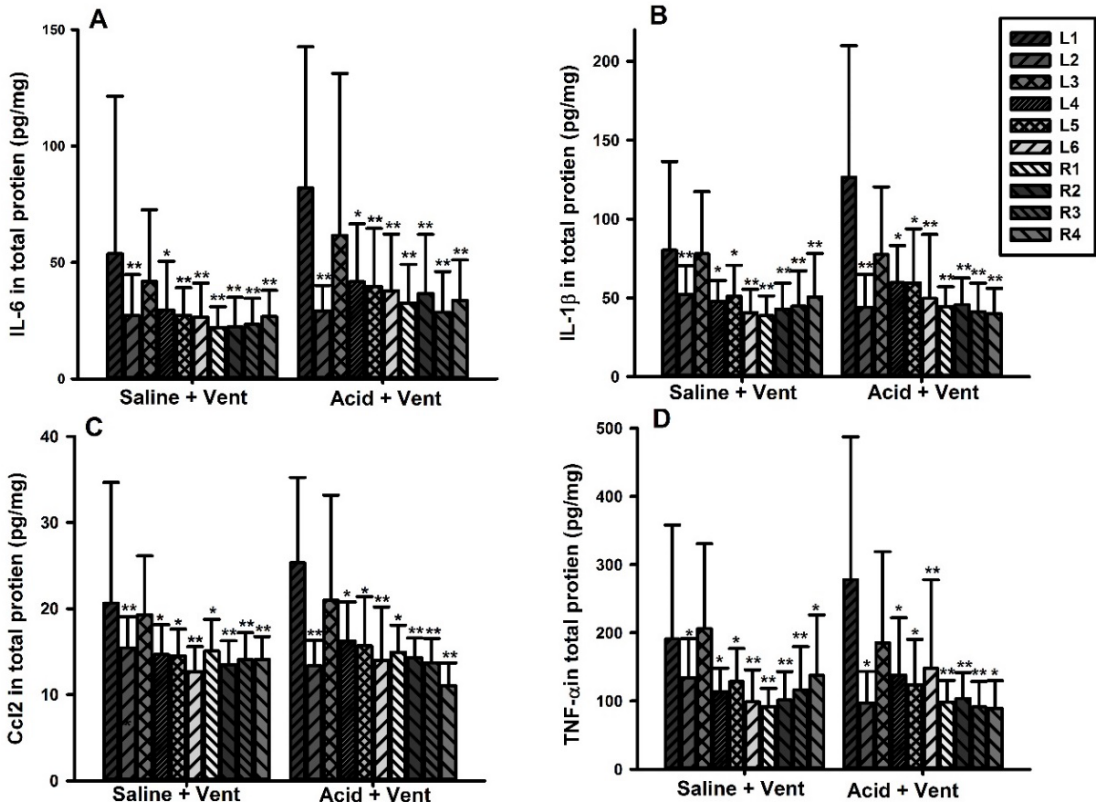
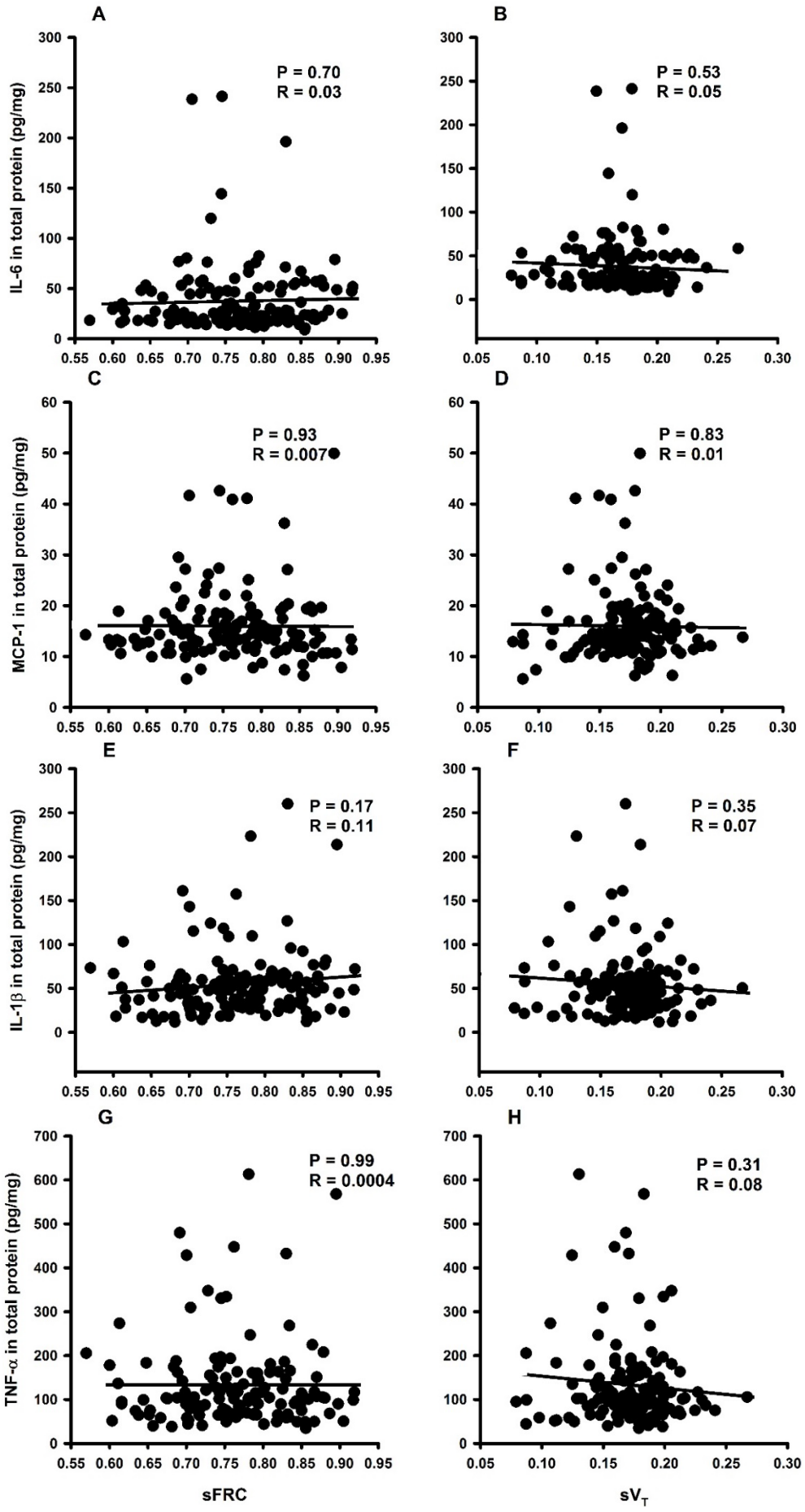


Figure 4.4: Regional protein levels in mechanically ventilated mice treated intratracheal acid or saline. Levels of the proteins IL-6 (A), IL-1β (B), Ccl2 (C), and TNF-α (D) in each lung region were measured by ELISA. The levels of the proteins are presented as a proportion of total protein, which was measured by Bradford assay. * and ** indicate $P < 0.05$ and $P < 0.001$ respectively relative to L1 within each group. N = 8 mice per group.

Figure 4.5: Relationship between regional protein levels and regional lung volumes (sFRC and sV_T at the onset of ventilation (H0) in ventilated mice (next page). Scatterplots showing the relationship between the regional sFRC (A, C, E and G) or sV_T (B, D, F and H) and regional levels of IL-6 (A and B), MCP-1 (C and D), IL-1β (E and F), and TNF-α (G and H) in the ventilated mice. Linear regression analysis was performed to assess the association and P-values are presented. Each dot represents a lung region from an individual mouse (N = 8 mice per group).


A
P = 0.70
R = 0.03
B
P = 0.53
R = 0.05
C
P = 0.93
R = 0.007
D
P = 0.83
R = 0.01
E
P = 0.17
R = 0.11
F
P = 0.35
R = 0.07
G
P = 0.99
R = 0.0004
H
P = 0.31
R = 0.08
IL-6 in total protein (pg/mg)
MCP-1 in total protein (pg/mg)
IL-1β in total protein (pg/mg)
TNF-α in total protein (pg/mg)
sFRC
sV$_T$

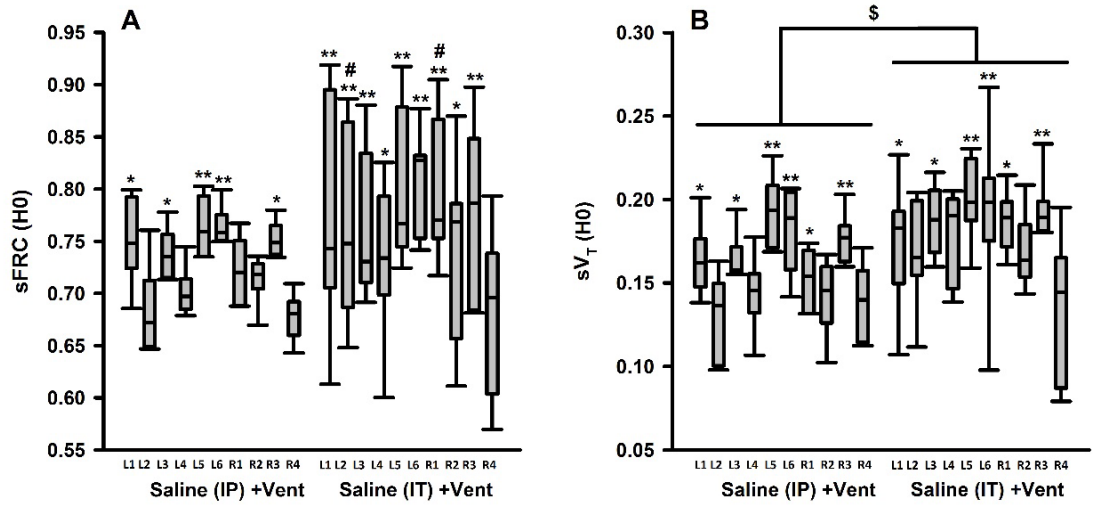


Figure 4.6: Effect of mode of saline administration (IP – Chapter 3 vs IT – Chapter 4) on lung volumes. Box plots (median, interquartile range, and 10th to 90th percentile) for sFRC and sV_T at baseline (panels A and B respectively) for each of ten lung regions in mice of each ventilated group. * and ** indicate $P < 0.05$ and $P < 0.001$ respectively relative to R4 within each group, # indicates $P < 0.05$ compared with same region in the IP injected group and $ indicates $P < 0.05$ between the two groups. N = 8 mice per group.

4.5 Discussion

In this Chapter, the aim was to assess the impact of mechanical ventilation on regional lung injury in a model of direct lung injury, by acid aspiration, to determine the effect of injury type on the response (compared to Chapter 3). We assessed regional lung volume by analysing high resolution dynamic 4D CT images, taken during ventilation, and measured the regional expression of inflammatory cytokines in lung tissues collected after 2 hours of ventilation. We found variations in regional lung volumes (sFRC and sV_T), but this was not altered by prior exposure to acid. Similarly, while there was significant regional variation in the expression of all the proteins measured, there was no effect of acid aspiration alone (free-breathing mice), mechanical ventilation alone (saline treated mice) or the combination (mechanical ventilation; saline vs acid) on cytokine expression. In order to further understand these observations, data from the IP saline group from Chapter 3 were compared with data from the IT saline group in this experiment. There was a significant effect of route of saline administration on the sFRC response whereby sFRC was increased in some regions while overall sV_T increased in the IT saline treated mice. It was clear that the lung volume estimates, particularly sFRC, were more variable in the control group compared to the control data from previous Chapters suggesting that the response was influenced by intratracheal liquid administration. Overall, the data showed no effect demonstrated for acid aspiration, mechanical ventilation or combination of both on regional lung response, which is likely due to the high pH of the acid and the effect of liquid aspiration on the regional lung response.

This is the first study to assess the impact of ventilation on regional cytokine expression in a model of acid aspiration. Analysis of the regional levels of the TNF-α, IL-1β, MCP-1 and IL-6 expression showed significant regional variation that was not

affected by prior treatment with acid aspiration. A further analysis to evaluate the impact of ventilation on the levels of measured proteins in the saline treated groups confirmed no significant impact of ventilation on the response. These results contradict the findings in the previous Chapters, in which ventilation was found to have an impact on the expression of these genes. These observations also contradict other studies where these proteins were shown to have altered expression in response to acid aspiration, ventilation or the combination of both [118, 148, 238, 251]. In the previous Chapters, regional mRNA levels of *Ccl2 (MCP-1)* and *IL-6* varied depending on ventilation strategy (Chapter 2) and pre-existing lung injury (Chapter 3). Differences in regional mRNA levels of *IL-1β* and *TNF-α* were also found in response to ventilation in the previous Chapters. Previous studies have found significant effects of acid aspiration on the expression these proteins [237-239, 242], but the acid was stronger with pH lower than 2.5, while increasing levels of these inflammatory protein in lung tissue, BAL or plasma have also been shown in response to mechanical ventilation [7, 118, 140, 148, 203].

The lack of an effect of acid aspiration or mechanical ventilation on the expression of the measured proteins in this Chapter may be due to several factors. First, the pH may have been too high. Because of ethical concerns, due to the impact on the welfare of the animals, it was not possible to use acid with pH lower than 3.0 as stipulated in our ethics approval. While it has been argued that a pH lower than 2.5 is required to induced significant injury to the lung [230], higher pH (4.0) have also been demonstrated to alter the lung response 2 hours after exposure [247]. This suggests the pH of the acid, used in this study, may be sufficient to induce injury, and a significant effect should have been found, especially in the ventilated mice where the response to the combination effects should be more severe [251, 252]. It is also possible that the duration of ventilation may not have been long enough for the response to develop, as

the second phase of injury, which is characterised by inflammation, in response to acid aspiration may take 3-4 hours to develop [230]. However, previous studies using 2 hours of ventilation have shown a significant effect [253, 254].

It possible that all of the above factors, combined with our early ventilation intervention with moderate PIP with PEEP was sufficient to prevent further injury [84]. For example, in a rat model of aspiration, after 2 hours ventilation with moderate V_T (< 15 mL/kg) and PEEP (2 cm H_2O), no additional effect on lung injury was observed [255]. The assessment of the inflammatory response by measuring the protein levels should also be acknowledged as a contributing factor. In the previous Chapters, gene expression was assessed by measuring mRNA levels, whereas in this Chapter protein levels were assessed. It is possible that there was an increase in gene expression, but sufficient time had not elapsed for this to translate into downstream changes in protein expression. Also, only four proteins were evaluated. A more robust assessment of a broader number of proteins may have provided additional insight. Overall, the results show neither acid nor ventilation had significant effect on the levels of the proteins that were measured. At this stage the reason for this regional variation are unclear but are likely to be linked to a combination of the relatively high pH and the short duration of ventilation using moderate PIP with PEEP.

The images analysis showed variation in regional lung volumes (sFRC and sV_T) with a similar pattern to that observed in previous Chapters. However, while there was an effect of endotoxemia (Chapter 3) on the regional lung volume response, particularly sFRC, the direct lung injury (acid aspiration) in this experiment had no effect on lung volumes. This was surprising because acid aspiration is well known to have deleterious effect on lung mechanics, such as increasing lung stiffness, airway resistance and inspiratory pressure response to mechanical ventilation [185, 247, 254, 256]. In

addition, there was no significant change in lung volume from baseline to 2 hours after ventilation suggesting no effect of both acid aspiration and ventilation on the lung volume response. These results contradict data from previous Chapters, in which ventilation was shown to influence the lung responses as demonstrated by the change in sFRC and sV_T from baseline to 2 hours after ventilation in both the healthy (Chapter 2) and pre-injured lung (Chapter 3).

Several factors may contribute to the lack of impact of both acid treatment and ventilation on the lung volume response in this experiment. For example, as indicated by the lack of change in the protein expression, the acid model used may not have been severe enough to induce lung injury. The result clearly shows that the instillation of liquid (saline and acid) had an impact on the inflation response of the lung to mechanical ventilation. This, combined with the uneven distribution of the fluid, is a likely explanation for the variability in the lung volumes that was observed. In order to further understand this issue, a further analysis was undertaken to assess the impact of liquid aspiration on the lung response by comparing lung volumes in saline treated mice with IT aspiration and IP injection (Chapter 3). The result showed, IT administration led to increases in regional sFRC, particularly in the upper dependent regions of the lung (L2 and R1). Regional sFRC was also highly variable, which is consistent with the effects of lung fluid and oedema formation [257] suggesting the effect of IT fluid instillation on the mechanical response of the lung. However, because the injury is mild, the increase in regional sFRC is more likely due to the effect of inhomogeneous fluid distribution on alveolar gas-trapping. Unfortunately, the regional variation of distribution of saline and acid instillation was not assessed. This may have provided some insight into the effect of fluid instillation on the variability in lung volumes that was observed.

It is interesting to note that there was also a higher overall sV_T in the IT saline group. Saline aspiration has been shown to increase alveolar surface tension [258] which is likely to decrease lung compliance leading to altered volume distribution. However, this is not consistent with the observation that sV_T increased in the IT saline group. There is no clear explanation for this discrepancy.

As detailed above, this experiment had several limitations which may have influenced the outcome. First, the pH of the acid used was likely too high to induce significant lung injury. This was due to a stipulation in our ethics approval on the advice of the animal welfare officer. In addition, the inflammatory assessment was limited to the levels of four proteins. It is possible that both acid aspiration and mechanical ventilation impacted on the expression of other proteins that are involved in lung injury and ventilator induced lung injury.

Overall, data of this Chapter showed acid aspiration, in combination with protective mechanical ventilation, had no effect on the lung response in this experiment. Although there was variation in regional lung volumes and level of inflammatory proteins there was no association between these measures. It is likely that the high pH and the effect of fluid administration on the lung mechanical response impacted on the outcomes in this experiment. Thus, we are unable to conclude whether acid aspiration and mechanical ventilation impacts on regional lung volumes and inflammation. Future studies should use a lower pH, a longer time between exposure to the acid and the onset of ventilation and measure a wider range of proteins.

Chapter 5 - General discussion

This study aimed to assess the impact of mechanical ventilation on the regional lung response and how this is altered by different types of pre-existing injured lung relevant to acute respiratory distress syndrome (ARDS). There were variations in the impact of mechanical ventilation on the regional lung response in the healthy lung (Chapter 2) and in the pre-injured endotoxemic lung (Chapter 3), but the effect of on the acid aspirated lung was inconclusive due to issues with the severity of the lung injury induced and the impact of fluid instillation (Chapter 4).

In the healthy lung (Chapter 2), there were heterogenous lung volume (sFRC, sV_T and distension) responses to mechanical ventilation whereby the regional sV_T and distension varied depending on ventilation strategy. In contrast, while sFRC also varied regionally, this variation was not influenced by the ventilation strategy. The expression of two key inflammatory genes, *IL-6* and *Ccl2*, varied regionally depending on the ventilation strategy, while the expression of other genes either varied regionally (*CxCl2*, *IL-1β, TNF-α, RAGE* and *Ang-2*) or in response to different ventilation strategies (*CxCl2*, *TNF-α, Wnt1, c-fos* and *Nfe2l2*) with no evidence of regional variation. Importantly, there were strong positive associations between regional expression of *IL-6, Ccl2,* and *Ang-2* and regional sV_T, but not sFRC, suggesting that regional over-inflation has the greatest impact on ventilation induced lung injury in the healthy lung.

In endotoxemia (Chapter 3), as a model of ARDS related pre-existing indirect lung injury, we found regional variation in the lung response to mechanical ventilation. Regional sFRC and the expression of *IL-6* and *Ccl2* varied depending on whether endotoxemia was present or not, while the regional sV_T varied independent of prior injury. The expression of *IL-6* in the endotoxemic mice was negatively associated with sFRC, whereas sV_T was not associated with the expression of any measured gene,

suggesting that, on contrast to the healthy lung, the endotoxemic lung is susceptible to low lung volume ventilation.

In acid aspiration (Chapter 4), as a model of direct lung injury, there was variation in regional lung volumes, however, acid aspiration had no effect on this response. The regional levels of inflammatory cytokines (TNF-α, MCP-1, IL-1β and IL-6) were not affected by acid aspiration alone (free-breathing mice), mechanical ventilation alone (saline treated mice) or the combination (within mechanical ventilation; saline vs acid). Follow up analysis comparing lung volume data of the mice treated with saline via IT instillation (Chapter 4) and IP injection (Chapter 3) demonstrated a significant effect of route of saline administration on the lung volume response. sFRC was increased in some regions while overall sV_T increased in the IT instillation group, suggesting the response was influenced by liquid aspiration. As a result, it was not possible to demonstrate the effect of acid aspiration on the lung volume response. In addition, prior acid-induced lung injury was not established, likely due to the high pH of the acid used, so the effect of mechanical ventilation on direct lung injury could not be assessed.

Overall, the impact of mechanical ventilation on the lung varied regionally and depended on both the ventilation strategy and pre-existing lung injury. The association between regional lung volume and the regionally inflammatory response suggests that the healthy lung is susceptible to over-stretch, while the endotoxemic lung is susceptible to low lung volume ventilation. These findings provide critical insight into the effect of mechanical ventilation on the regional lung response and may inform future work that can potentially be translated into beneficial outcomes in patients with ARDS.

One of the primary findings of this study was a heterogenous response of healthy lung to mechanical ventilation. In a clinical context, mechanical ventilation is often used to support respiration in the absence of significant prior lung injury. For example, the development of the modern positive pressure ventilation in the ICU started during the poliomyelitis epidemic in 1952, in which the ventilation was used to save thousands of patients with otherwise “healthy” lungs [8, 66]. While polio may have been eradicated in most countries, the application of mechanical ventilation in patients who require ventilatory support as a result of impaired respiratory drive, especially in anesthesia, is still an important mode of treatment [6]. However, this can be problematic because mechanical ventilation is well known to cause injury in an otherwise healthy lung [10, 68].

Ventilation related mortality in anesthesia had reduced from about a quarter of mortality in anesthesia in the 1980s to 10% by 2001 [259-261]. As a result of the ARDSNet study [57], which showed a positive benefit of reduced tidal volume in patients with respiratory failure, applied tidal volumes have reduced with a view to improving these mortality rates further. Nevertheless, clinicians have been hesitant to apply this protective ventilation strategy in anesthesia because of concerns about hypoxia due to atelectasis [262-264]. This was supported by a retrospective patient review study which showed an association between low PEEP ventilation and increased risk of 30-day mortality [265]. However, a recent systematic review reported no significant effect of different ventilation strategies on mortality and length of stay in patients without acute lung injury [266] suggesting that the concerns regarding low PEEP may be unwarranted.

The data presented in Chapter 2, where the response in the healthy lung was assessed, clearly showed that the response within the healthy lung is heterogeneous. The

heterogenous lung response was characterized by variation in sV_T and expression of *IL-6* and *Ccl2*. The positive association between these outcomes highlights the importance of regional overstretch on the lung injury response in the healthy lung and the importance of considering what is occurring at a regional level. The lack of association between sFRC and gene expression suggests that cyclic stretch is not as critical as overdistension in the healthy lung setting which supports the concept that high tidal volumes should be avoided in the clinical application.

In contrast to the healthy lung, where regional overdistension had a detrimental effect on the lung response, the endotoxemic lung appeared to be susceptible to low lung volume ventilation. Sepsis, a major risk factor for ARDS development, is associated with the most severe forms of ARDS and accounts for the highest patient mortality [23, 24]. While the majority of mortality in patients with ARDS is due to multiple system organ failure (MSOF) [43, 44], potentially as a result of ventilator-induced lung injury (VILI) [8, 9], the mechanisms of VILI development in the septic lung are poorly understood [6].

Over the last decade, studies have been focusing on reducing VILI in patients with ARDS to improve mortality, however, defining a standard ventilation strategy for the injured lung has been problematic as the proportion of relatively “inflatable” regions of the lung varies between patients [16]. The relative proportion of healthy lung in patients with severe ARDS may be as low as 20% [17, 46], with the bulk of the lung being collapsed or fluid filled. In order to avoid VILI, lower tidal volumes may be required to ventilate the small regions of health lung as demonstrated by the ARDSNet study [57]. However, since the ARDSNet study, mortality rates in ARDS patients have remains unchanged [3] and there is some concern that low lung volume ventilation may also contribute to mortality in these patients [267, 268].

The ARDSNet study suggested a V_T of 6 mL/kg [57] or less, but this low V_T was shown to induce more harm than 10 mL/kg in patients with relatively compliant lungs [267, 268]. Similarly, higher levels of PEEP were found to be associated with better survival in with severe ARDS [59] but clinical trials to assess the benefit of PEEP adjustment showed no effect [62]. As a result, the ARDS definition taskforce recommended a V_T of 6-8 mL/kg and $\geq$ 10 cm H_2O PEEP [4]. However, a recent clinical study reported that median tidal volumes > 8 mL/kg were not associated with increased mortality [269] while, a recent review article suggested different levels of PEEP should be applied depending on disease severity [270]. The relative contribution of over and under-ventilation to the inflammatory response in the ARDS lung remains unclear.

Chapter 3 provides critical insight into the regional response of the endotoxemic lung to a protective ventilation strategy with relatively low tidal volume and moderate PEEP. It was clear that endotoxemia altered end-tidal lung volumes but had no effect of V_T. The strong negative association between regional sFRC and the expression of *IL-6*, and no significant association between sV_T and gene expression, highlights the importance of regional low lung volume ventilation in contributing to lung injury in endotoxemia. This supports the clinical concern regarding the impact of under-ventilation on VILI in sepsis induced ARDS and highlights the importance of considering the regional response.

This study had several limitations which should be acknowledged. First, this study was conducted entirely in a small animal model, which may not be representative of the clinical response in humans. However, this model allowed measurement of the heterogenous response of the lung, the application of an injurious ventilation strategy and the controlled induction of prior injury. The fact that the markers that were

consistently expressed (e.g. *IL-6*) are also linked to mortality in patients with ARDS [37, 42, 138] supports the clinical relevance of this work. In addition, demonstration of the application of dynamic 4D CT imaging may provide a path forward for improvements in monitoring the response to mechanical ventilation in critically ill patients to optimize ventilation strategies by considering the regional lung response in an individual patient

Another limitation of this study was the apparent lack of injury induced by acid aspiration in Chapter 4 which meant that the effect of direct lung injury on the response could not be determined. Since ARDS development is caused by a wide range of risk factors, further study in different injury models may be required. In future studies a lower pH and a longer time between acid exposure and mechanical ventilation may provide further insight - noting that animal welfare will be an important consideration.

The short ventilation period (2 hours) applied in this study is also a limitation as longer ventilation periods are typically applied in the clinic and may last several days [271]. This ventilation duration may be too short for many of the biological and lung structural responses to develop. However, while the ventilation period applied in this study may have been too short, a significant effect on the regional lung response was observed and there was evidence of upregulation of inflammatory markers that are seen clinically (Chapter 2). Similarly, prior injury clearly modified the response to this short duration of mechanical ventilation (Chapter 3).

The assessment of the inflammatory response was also limited to selected genes (Chapter 2 and 3) and proteins (Chapter 4). Further study to assess the expression of a wider range of pathways that may be involved in ventilator induced lung injury may

provide further insight. Likewise, measurement of the systemic response is also critical to understand the link between the lung response and development of MSOF

In conclusion, this Thesis has shown that mechanical ventilation can induce regional inflammation in the healthy lung as a result of overdistension. In contrast, variation in the regional inflammatory response in the endotoxemic lung was influenced by low regional end expiratory lung volume. It is well known that VILI can occur through either overdistension (volutrauma) or cyclic stretch (atelectrauma). However, until now, it was unclear which was the most important as they both can occur within the same lung. The findings of this Thesis contribute to a greater understanding of the mechanisms of VILI and how the lung response varies depending on ventilation strategy and pre-existing lung injury.

In the clinical context, mechanical ventilation is a lifesaving therapy in patients with respiratory failure. Data from this Thesis show that different ventilation strategies may be required for patients with different lung conditions. However, the lack of a well-defined clinical tool to monitor the regional lung response at the beside remains an issue. In addition, the impact of mechanical ventilation on the lung in the setting of different injury types requires further work. Therefore, future studies to assess the impact of ventilation on the response in different types of prior lung injury and the further development of clinical tool to characterize the regional response at the bedside may provide the opportunity to improve outcomes in these critically ill patients.

The following appendix has been removed for copyright or proprietary reasons.

It is: Yen, S., Preissner M., Bennett E., Dubsky, S., Carnibella, R., O'Toole, R., Roddam, L., Jones, H., Dargaville, P. A., Fouras, A. Zosky, G. R., 2019. The link between regional tidal stretch and lung injury during mechanical ventilation, American journal of respiratory cell and molecular biology, 60(5), 569-577

References

1. Burt, C.C. and Arrowsmith, J.E. (**2009**). Respiratory failure. *Surgery (Oxford)* **27**(11): p. 475-479.
2. Hart, N. (**2008**). Respiratory failure. *Medicine* **36**(5): p. 242-245.
3. Villar, J., Blanco, J., and Kacmarek, R.M. (**2016**). Current incidence and outcome of the acute respiratory distress syndrome. *Current Opinion in Critical Care* **22**(1): p. 1-6.
4. Ranieri, V.M., Rubenfeld, G.D., Thompson, B.T., Ferguson, N.D., Caldwell, E., Fan, E., Camporota, L., and Slutsky, A.S. (**2012**). Acute respiratory distress syndrome: the Berlin Definition. *Journal of the American Medical Association* **307**(23): p. 2526-33.
5. Bellani, G., Laffey, J.G., Pham, T., Fan, E., Brochard, L., Esteban, A., Gattinoni, L., van Haren, F., Larsson, A., McAuley, D.F., Ranieri, M., Rubenfeld, G., Thompson, B.T., Wrigge, H., Slutsky, A.S., and Pesenti, A. (**2016**). Epidemiology, patterns of care, and mortality for patients with acute respiratory distress syndrome in intensive care units in 50 countries. *Journal of the American Medical Association* **315**(8): p. 788-800.
6. Slutsky, A.S. and Ranieri, V.M. (**2013**). Ventilator-induced lung injury. *New England Journal of Medicine* **369**(22): p. 2126-2136.
7. Imai, Y., Parodo, J., Kajikawa, O., de Perrot, M., Fischer, S., Edwards, V., Cutz, E., Liu, M., Keshavjee, S., Martin, T.R., Marshall, J.C., Ranieri, V.M., and Slutsky, A.S. (**2003**). Injurious mechanical ventilation and end-organ epithelial cell apoptosis and organ dysfunction in an experimental model of

acute respiratory distress syndrome. *Journal of the American Medical Association* **289**(16): p. 2104-12.

8. Slutsky, A.S. (**2005**). Ventilator-induced lung injury: from barotrauma to biotrauma. *Respiratory Care* (5): p. 646-659.
9. Dreyfuss, D. and Saumon, G. (**1998**). Ventilator-induced lung injury. *American Journal of Respiratory and Critical Care Medicine* **157**(1): p. 294-323.
10. Dreyfuss, D., Soler, P., Basset, G., and Saumon, G. (**1988**). High inflation pressure pulmonary edema: respective effects of high airway pressure, high tidal volume, and positive end-expiratory pressure. *American Review of Respiratory Disease* **137**(5): p. 1159-1164.
11. Muscedere, J.G., Mullen, J.B., Gan, K., and Slutsky, A.S. (**1994**). Tidal ventilation at low airway pressures can augment lung injury. *American Journal of Respiratory and Critical Care Medicine* **149**(5): p. 1327-1334.
12. Thompson, B.T., Chambers, R.C., and Liu, K.D. (**2017**). Acute respiratory distress syndrome. *The New England Journal of Medicine* **377**(6): p. 562-572.
13. Cressoni, M., Chiurazzi, C., Gotti, M., Amini, M., Brioni, M., Algieri, I., Cammaroto, A., Rovati, C., Massari, D., di Castiglione, C.B., Nikolla, K., Montaruli, C., Lazzerini, M., Dondossola, D., Colombo, A., Gatti, S., Valerio, V., Gagliano, N., Carlesso, E., and Gattinoni, L. (**2015**). Lung inhomogeneities and time course of ventilator-induced mechanical injuries. *Anesthesiology* **123**(3): p. 618-27.
14. Paula, L.F., Wellman, T.J., Winkler, T., Spieth, P.M., Güldner, A., Venegas, J.G., Gama de Abreu, M., Carvalho, A.R., and Vidal Melo, M.F. (**2016**). Regional tidal lung strain in mechanically ventilated normal lungs. *Journal of Applied Physiology* **121**(6): p. 1335-1347.

15. Dargaville, P., Rimensberger, P., and Frerichs, I. (**2010**). Regional tidal ventilation and compliance during a stepwise vital capacity manoeuvre. *Intensive Care Medicine* **36**(11): p. 1953-1961.

16. Cressoni, M., Cadringher, P., Chiurazzi, C., Amini, M., Gallazzi, E., Marino, A., Brioni, M., Carlesso, E., Chiumello, D., Quintel, M., Bugedo, G., and Gattinoni, L. (**2014**). Lung inhomogeneity in patients with acute respiratory distress syndrome. *American Journal of Respiratory and Critical Care Medicine* **189**(2): p. 149-58.

17. Gattinoni, L., Marini, J.J., Pesenti, A., Quintel, M., Mancebo, J., and Brochard, L. (**2016**). The "baby lung" became an adult. *Intensive Care Medicine* **42**(5): p. 663-73.

18. De Prost, N., Ricard, J.-D., Saumon, G., and Dreyfuss, D. (**2011**). Ventilator-induced lung injury: historical perspectives and clinical implications. *Annals of Intensive Care* **1**(1): p. 28.

19. Ostwani, W. and Shanley, T.P. (**2014**). Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), in *Pediatric Critical Care Medicine: Volume 2: Respiratory, Cardiovascular and Central Nervous Systems*, Wheeler, S.D., Wong, R.H., and Shanley, P.T., Editors. Springer London: London. p. 101-126.

20. Ashbaugh, D.G., Boyd Bigelow, D., Petty, T., and Levine, B. (**1967**). Acute respiratory distress in adults. *The Lancet* **290**(7511): p. 319-323.

21. Ware, L.B. and Matthay, M.A. (**2000**). The acute respiratory distress syndrome. *The New England Journal of Medicine* **342**(18): p. 1334-49.

22. Bernard, G.R., Artigas, A., Brigham, K.L., Carlet, J., Falke, K., Hudson, L., Lamy, M., LeGall, J.R., Morris, A., Spragg, R., and Consensus, C. (**1994**). Report of the American-European Consensus Conference on acute respiratory

distress syndrome: Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Journal of Critical Care* **9**(1): p. 72-81.

23. Rubenfeld, G.D., Caldwell, E., Peabody, E., Weaver, J., Martin, D.P., Neff, M., Stern, E.J., and Hudson, L.D. (**2005**). Incidence and outcomes of acute lung injury. *New England Journal of Medicine* **353**(16): p. 1685-1693.

24. Brun-Buisson, C., Minelli, C., Bertolini, G., Brazzi, L., Pimentel, J., Lewandowski, K., Bion, J., Romand, J.-A., Villar, J., Thorsteinsson, A., Damas, P., Armaganidis, A., and Lemaire, F. (**2003**). Epidemiology and outcome of acute lung injury in European intensive care units. *Intensive Care Medicine* **30**(1): p. 51-61.

25. Hudson, L.D., Milberg, J.A., Anardi, D., and Maunder, R.J. (**1995**). Clinical risks for development of the acute respiratory distress syndrome. *American Journal of Respiratory and Critical Care Medicine* **151**(2 Pt 1): p. 293-301.

26. Doyle, R.L., Szaflarski, N., Modin, G.W., Wiener-Kronish, J.P., and Matthay, M.A. (**1995**). Identification of patients with acute lung injury. Predictors of mortality. *American Journal of Respiratory and Critical Care Medicine* **152**(6 Pt 1): p. 1818-24.

27. Chen, W., Chen, Y.Y., Tsai, C.F., Chen, S.C., Lin, M.S., Ware, L.B., and Chen, C.M. (**2015**). Incidence and outcomes of acute respiratory distress syndrome: A nationwide registry-based study in Taiwan, 1997 to 2011. *Medicine (Baltimore)* **94**(43): p. e1849.

28. Caser, E.B., Zandonade, E., Pereira, E., Gama, A.M., and Barbas, C.S. (**2014**). Impact of distinct definitions of acute lung injury on its incidence and outcomes in Brazilian ICUs: prospective evaluation of 7,133 patients. *Critical Care Medicine* **42**(3): p. 574-82.

29. Bhadade, R.R., de Souza, R.A., Harde, M.J., and Khot, A. (**2011**). Clinical characteristics and outcomes of patients with acute lung injury and ARDS. *Journal of Postgraduate Medicine* **57**(4): p. 286-90.

30. Seeley, E., McAuley, D.F., Eisner, M., Miletin, M., Matthay, M.A., and Kallet, R.H. (**2008**). Predictors of mortality in acute lung injury during the era of lung protective ventilation. *Thorax* **63**(11): p. 994-8.

31. Zambon, M. and Vincent, J.-L. (**2008**). Mortality rates for patients with acute lung injury/ards have decreased over time. *Chest* **133**(5): p. 1120-1127.

32. Phua, J., Badia, J.R., Adhikari, N.K.J., Friedrich, J.O., Fowler, R.A., Singh, J.M., Scales, D.C., Stather, D.R., Li, A., Jones, A., Gattas, D.J., Hallett, D., Tomlinson, G., Stewart, T.E., and Ferguson, N.D. (**2009**). Has mortality from acute respiratory distress syndrome decreased over time? *American Journal of Respiratory and Critical Care Medicine* **179**(3): p. 220-227.

33. Villar, J., Sulemanji, D., and Kacmarek, R.M. (**2014**). The acute respiratory distress syndrome: incidence and mortality, has it changed? *Current Opinion in Critical Care* **20**(1): p. 3-9.

34. Lee, C.T., Fein, A.M., Lippmann, M., Holtzman, H., Kimbel, P., and Weinbaum, G. (**1981**). Elastolytic activity in pulmonary lavage fluid from patients with adult respiratory-distress syndrome. *The New England Journal of Medicine* **304**(4): p. 192-6.

35. Meade, P., Shoemaker, W.C., Donnelly, T.J., Abraham, E., Jagels, M.A., Cryer, H.G., Hugli, T.E., Bishop, M.H., and Wo, C.C. (**1994**). Temporal patterns of hemodynamics, oxygen transport, cytokine activity, and complement activity in the development of adult respiratory distress syndrome after severe injury. *The Journal of Trauma* **36**(5): p. 651-7.

36. Damas, P., Reuter, A., Gysen, P., Demonty, J., Lamy, M., and Franchimont, P. (**1989**). Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans. *Critical Care Medicine* **17**(10): p. 975-8.

37. Calandra, T., Gerain, J., Heumann, D., Baumgartner, J.D., and Glauser, M.P. (**1991**). High circulating levels of interleukin-6 in patients with septic shock: evolution during sepsis, prognostic value, and interplay with other cytokines. *The American Journal of Medicine* **91**(1): p. 23-9.

38. Siler, T.M., Swierkosz, J.E., Hyers, T.M., Fowler, A.A., and Webster, R.O. (**1989**). Immunoreactive interleukin-1 in bronchoalveolar lavage fluid of high-risk patients and patients with the adult respiratory distress syndrome. *Experimental Lung Research* **15**(6): p. 881-94.

39. Jacobs, R.F., Tabor, D.R., Burks, A.W., and Campbell, G.D. (**1989**). Elevated interleukin-1 release by human alveolar macrophages during the adult respiratory distress syndrome. *The American Review of Respiratory Disease* **140**(6): p. 1686-92.

40. Jorens, P.G., Van Damme, J., De Backer, W., Bossaert, L., De Jongh, R.F., Herman, A.G., and Rampart, M. (**1992**). Interleukin 8 (IL-8) in the bronchoalveolar lavage fluid from patients with the adult respiratory distress syndrome (ARDS) and patients at risk for ARDS. *Cytokine* **4**(6): p. 592-7.

41. Chollet-Martin, S., Montravers, P., Gibert, C., Elbim, C., Desmonts, J.M., Fagon, J.Y., and Gougerot-Pocidalo, M.A. (**1993**). High levels of interleukin-8 in the blood and alveolar spaces of patients with pneumonia and adult respiratory distress syndrome. *Infection and Immunity* **61**(11): p. 4553-9.

42. Meduri, G.U., Kohler, G., Headley, S., Tolley, E., Stentz, F., and Postlethwaite, A. (**1995**). Inflammatory cytokines in the BAL of patients with ARDS.

Persistent elevation over time predicts poor outcome. *Chest* **108**(5): p. 1303-14.

43. Hudson, L.D. (**1989**). Survival data in patients with acute and chronic lung disease requiring mechanical ventilation. *The Amercan Review of Respiratory Disease* **140**(2): p. S19-24.

44. Montgomery, A.B., Stager, M.A., Carrico, C.J., and Hudson, L.D. (**1985**). Causes of mortality in patients with the adult respiratory distress syndrome. *The American Review of Respiratory Disease* **132**(3): p. 485-9.

45. Gattinoni, L., Mascheroni, D., Torresin, A., Marcolin, R., Fumagalli, R., Vesconi, S., Rossi, G.P., Rossi, F., Baglioni, S., Bassi, F., and et al. (**1986**). Morphological response to positive end expiratory pressure in acute respiratory failure. Computerized tomography study. *Intensive Care Medicine* **12**(3): p. 137-42.

46. Gattinoni, L. and Pesenti, A. (**2005**). The concept of "baby lung". *Intensive Care Medicine* **31**(6): p. 776-784.

47. Gattinoni, L. and Pesenti, A. (**1987**). ARDS: the non-homogeneous lung; facts and hypothesis. *Intensive and Critical Care Digest* **6**(1): p. 1-4.

48. Gattinoni, L. and Quintel, M. (**2016**). Is mechanical ventilation a cure for ARDS? *Intensive Care Medicine* **42**(5): p. 916-917.

49. Plotz, F.B., Slutsky, A.S., van Vught, A.J., and Heijnen, C.J. (**2004**). Ventilator-induced lung injury and multiple system organ failure: a critical review of facts and hypotheses. *Intensive Care Medicine* **30**(10): p. 1865-72.

50. Brochard, L., Mancebo, J., Wysocki, M., Lofaso, F., Conti, G., Rauss, A., Simonneau, G., Benito, S., Gasparetto, A., Lemaire, F., and et al. (**1995**). Noninvasive ventilation for acute exacerbations of chronic obstructive

pulmonary disease. *The New England Journal of Medicine* **333**(13): p. 817-22.

51. Li, G., Malinchoc, M., Cartin-Ceba, R., Venkata, C.V., Kor, D.J., Peters, S.G., Hubmayr, R.D., and Gajic, O. (**2011**). Eight-year trend of acute respiratory distress syndrome: a population-based study in Olmsted County, Minnesota. *American Journal of Respiratory and Critical Care Medicine* **183**(1): p. 59-66.

52. Andrews, P.L., Sadowitz, B., Kollisch-Singule, M., Satalin, J., Roy, S., Snyder, K., Gatto, L.A., Nieman, G.F., and Habashi, N.M. (**2015**). Alveolar instability (atelectrauma) is not identified by arterial oxygenation predisposing the development of an occult ventilator-induced lung injury. *Intensive Care Medicine Experimental* **3**(1): p. 16.

53. Kumar, A., Pontoppidan, H., Falke, K.J., Wilson, R.S., and Laver, M.B. (**1973**). Pulmonary barotrauma during mechanical ventilation. *Critical Care Medicine* **1**(4): p. 181-6.

54. Nennhaus, H.P., Javid, H., and Julian, O.C. (**1967**). Alveolar and pleural rupture. Hazards of positive pressure respiration. *Archives of Surgery* **94**(1): p. 136-41.

55. Kolobow, T., Moretti, M.P., Fumagalli, R., Mascheroni, D., Prato, P., Chen, V., and Joris, M. (**1987**). Severe impairment in lung function induced by high peak airway pressure during mechanical ventilation. An experimental study. *The American Review of Respiratory Disease* **135**(2): p. 312-5.

56. Protti, A., Cressoni, M., Santini, A., Langer, T., Mietto, C., Febres, D., Chierichetti, M., Coppola, S., Conte, G., Gatti, S., Leopardi, O., Masson, S., Lombardi, L., Lazzerini, M., Rampoldi, E., Cadringher, P., and Gattinoni, L. (**2011**). Lung stress and strain during mechanical ventilation: any safe

threshold? *American Journal of Respiratory and Critical Care Medicine* **183**(10): p. 1354-62.

57. Brower, R.G., Matthay, M.A., Morris, A., Schoenfeld, D., Thompson, B.T., and Wheeler, A. (**2000**). Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *The New England Journal of Medicine* **342**(18): p. 1301-8.

58. Schultz, M.J. (**2008**). Lung-protective mechanical ventilation with lower tidal volumes in patients not suffering from acute lung injury: a review of clinical studies. *Medical Science Monitor* **14**(2): p. Ra22-26.

59. Briel, M., Meade, M., Mercat, A., Brower, R.G., Talmor, D., Walter, S.D., Slutsky, A.S., Pullenayegum, E., Zhou, Q., Cook, D., Brochard, L., Richard, J.C., Lamontagne, F., Bhatnagar, N., Stewart, T.E., and Guyatt, G. (**2010**). Higher vs lower positive end-expiratory pressure in patients with acute lung injury and acute respiratory distress syndrome: systematic review and meta-analysis. *Journal of the American Medical Association* **303**(9): p. 865-73.

60. Meadows, S.K., Dressman, H.K., Muramoto, G.G., Himburg, H., Salter, A., Wei, Z., Ginsburg, G., Chao, N.J., Nevins, J.R., and Chute, J.P. (**2008**). Gene expression signatures of radiation response are specific, durable and accurate in mice and humans. *PLoS ONE* **3**(4): p. e1912.

61. Mercat, A., Richard, J.C., Vielle, B., Jaber, S., Osman, D., Diehl, J.L., Lefrant, J.Y., Prat, G., Richecoeur, J., Nieszkowska, A., Gervais, C., Baudot, J., Bouadma, L., and Brochard, L. (**2008**). Positive end-expiratory pressure setting in adults with acute lung injury and acute respiratory distress syndrome: a randomized controlled trial. *Journal of the American Medical Association* **299**(6): p. 646-55.

62. Brower, R.G., Lanken, P.N., MacIntyre, N., Matthay, M.A., Morris, A., Ancukiewicz, M., Schoenfeld, D., and Thompson, B.T. (**2004**). Higher versus lower positive end-expiratory pressures in patients with the acute respiratory distress syndrome. *The New England Journal of Medicine* **351**(4): p. 327-36.

63. Meade, M.O., Cook, D.J., Guyatt, G.H., and et al. (**2008**). Ventilation strategy using low tidal volumes, recruitment maneuvers, and high positive end-expiratory pressure for acute lung injury and acute respiratory distress syndrome: A randomized controlled trial. *Journal of the American Medical Association* **299**(6): p. 637-645.

64. Mead, J., Takishima, T., and Leith, D. (**1970**). Stress distribution in lungs: a model of pulmonary elasticity. *Journal of Applied Physiology* **28**(5): p. 596-608.

65. Hernandez, L.A., Coker, P.J., May, S., Thompson, A.L., and Parker, J.C. (**1990**). Mechanical ventilation increases microvascular permeability in oleic acid-injured lungs. *Journal of Applied Physiology* **69**(6): p. 2057-61.

66. Lassen, H. (**1953**). A preliminary report on the 1952 poliomyelitis epidemic in. *Classic Papers in Critical Care* **1**: p. 1.

67. Bersten, A., Edibam, C., Hunt, T., Moran, J., and Group, T.I. (**2002**). Incidence and mortality of acute lung injury and the acute respiratory distress syndrome in three Australian states. *American Journal of Respiratory and Critical Care Medicine* **165**(4): p. 443-448.

68. Webb, H.H. and Tierney, D.F. (**1974**). Experimental pulmonary edema due to intermittent positive pressure ventilation with high inflation pressures. protection by positive end-expiratory pressure. *American Review of Respiratory Disease* **110**(5): p. 556-565.

69. Greenfield, L.J., Ebert, P.A., and Benson, D.W. (**1964**). Effect of positive pressure ventilation on surface tension properties of ling extracts. *Anesthesiology* **25**: p. 312-6.

70. Dreyfuss, D., Basset, G., Soler, P., and Saumon, G. (**1985**). Intermittent positive-pressure hyperventilation with high inflation pressures produces pulmonary microvascular injury in rats. *The American Review of Respiratory Disease* **132**(4): p. 880-4.

71. Parker, J.C., Hernandez, L.A., Longenecker, G.L., Peevy, K., and Johnson, W. (**1990**). Lung edema caused by high peak inspiratory pressures in dogs. Role of increased microvascular filtration pressure and permeability. *The American Review of Respiratory Disease* **142**(2): p. 321-8.

72. Caironi, P., Ichinose, F., Liu, R., Jones, R.C., Bloch, K.D., and Zapol, W.M. (**2005**). 5-Lipoxygenase deficiency prevents respiratory failure during ventilator-induced lung injury. *American Journal of Respiratory and Critical Care Medicine* **172**(3): p. 334-43.

73. Gattinoni, L., Protti, A., Caironi, P., and Carlesso, E. (**2010**). Ventilator-induced lung injury: the anatomical and physiological framework. *Critical Care Medicine* **38**(10 Suppl): p. S539-48.

74. Villar, J., Herrera-Abreu, M.T., Valladares, F., Muros, M., Perez-Mendez, L., Flores, C., and Kacmarek, R.M. (**2009**). Experimental ventilator-induced lung injury: exacerbation by positive end-expiratory pressure. *Anesthesiology* **110**(6): p. 1341-7.

75. Walder, B., Fontao, E., Totsch, M., and Morel, D.R. (**2005**). Time and tidal volume-dependent ventilator-induced lung injury in healthy rats. *European Journal of Anaesthesiology* **22**(10): p. 785-94.

76. Wilson, M.R., Choudhury, S., Goddard, M.E., O'Dea, K.P., Nicholson, A.G., and Takata, M. (**2003**). High tidal volume upregulates intrapulmonary cytokines in an in vivo mouse model of ventilator-induced lung injury. *Journal of Applied Physiology* **95**(Journal of Applied Psychology4): p. 1385-93.

77. Tschumperlin, D.J. and Margulies, S.S. (**1999**). Alveolar epithelial surface area-volume relationship in isolated rat lungs. *Journal of Applied Physiology* **86**(6): p. 2026-33.

78. Wirtz, H.R. and Dobbs, L.G. (**1990**). Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells. *Science* **250**(4985): p. 1266-9.

79. Scott, J.E., Yang, S.Y., Stanik, E., and Anderson, J.E. (**1993**). Influence of strain on [3H]thymidine incorporation, surfactant-related phospholipid synthesis, and cAMP levels in fetal type II alveolar cells. *American Journal of Respiratory Cell and Molecular Biology* **8**(3): p. 258-65.

80. Tschumperlin, D.J. and Margulies, S.S. (**1998**). Equibiaxial deformation-induced injury of alveolar epithelial cells in vitro. *The American Journal of Physiology* **275**(6 Pt 1): p. L1173-83.

81. Cavanaugh, K.J., Cohen, T.S., and Margulies, S.S. (**2006**). Stretch increases alveolar epithelial permeability to uncharged micromolecules. *American Journal of Physiology. Cell Physiology* **290**(4): p. C1179-88.

82. Cavanaugh, K.J., Jr., Oswari, J., and Margulies, S.S. (**2001**). Role of stretch on tight junction structure in alveolar epithelial cells. *American Journal of Respiratory Cell and Molecular Biology* **25**(5): p. 584-91.

83. Hamlington, K.L., Ma, B., Smith, B.J., and Bates, J.H. (**2016**). Modeling the progression of epithelial leak caused by overdistension. *Cellular and Molecular Bioengineering* **9**(1): p. 151-161.

84. Zosky, G.R., Cannizzaro, V., Hantos, Z., and Sly, P.D. (**2009**). Protective mechanical ventilation does not exacerbate lung function impairment or lung inflammation following influenza A infection. *Journal of Applied Physiology* **107**(5): p. 1472-1478.

85. Slutsky, A.S. (**1999**). Lung injury caused by mechanical ventilation. *Chest* **116**(suppl_1): p. 9S-15S.

86. Lachmann, B., Jonson, B., Lindroth, M., and Robertson, B. (**1982**). Modes of artificial ventilation in severe respiratory distress syndrome. Lung function and morphology in rabbits after wash-out of alveolar surfactant. *Critical Care Medicine* **10**(11): p. 724-32.

87. Hamilton, P.P., Onayemi, A., Smyth, J.A., Gillan, J.E., Cutz, E., Froese, A.B., and Bryan, A.C. (**1983**). Comparison of conventional and high-frequency ventilation: oxygenation and lung pathology. *Journal of Applied Physiology: Respiratory Environmental and Exercise Physiology* **55**(1 Pt 1): p. 131-8.

88. Kolton, M., Cattran, C.B., Kent, G., Volgyesi, G., Froese, A.B., and Bryan, A.C. (**1982**). Oxygenation during high-frequency ventilation compared with conventional mechanical ventilation in two models of lung injury. *Anesthesia and Analgesia* **61**(4): p. 323-32.

89. McCulloch, P.R., Forkert, P.G., and Froese, A.B. (**1988**). Lung volume maintenance prevents lung injury during high frequency oscillatory ventilation in surfactant-deficient rabbits. *The American Review of Respiratory Disease* **137**(5): p. 1185-92.

90. Nash, G., Bowen, J.A., and Langlinais, P.C. (**1971**). "Respirator lung": a misnomer. *Archives of Pathology (Chicago)* **91**(3): p. 234-40.

91. D'Angelo, E., Pecchiari, M., Baraggia, P., Saetta, M., Balestro, E., and Milic-Emili, J. (**2002**). Low-volume ventilation causes peripheral airway injury and

increased airway resistance in normal rabbits. *Journal of Applied Physiology* **92**(3): p. 949-56.

92. D'Angelo, E., Pecchiari, M., Saetta, M., Balestro, E., and Milic-Emili, J. (**2004**). Dependence of lung injury on inflation rate during low-volume ventilation in normal open-chest rabbits. *Journal of Applied Physiology* **97**(1): p. 260-8.

93. D'Angelo, E., Pecchiari, M., Della Valle, P., Koutsoukou, A., and Milic-Emili, J. (**2005**). Effects of mechanical ventilation at low lung volume on respiratory mechanics and nitric oxide exhalation in normal rabbits. *Journal of Applied Physiology* **99**(2): p. 433-44.

94. David, M., Karmrodt, J., Bletz, C., David, S., Herweling, A., Kauczor, H.U., and Markstaller, K. (**2005**). Analysis of atelectasis, ventilated, and hyperinflated lung during mechanical ventilation by dynamic CT. *Chest* **128**(5): p. 3757-70.

95. Markstaller, K., Eberle, B., Kauczor, H.U., Scholz, A., Bink, A., Thelen, M., Heinrichs, W., and Weiler, N. (**2001**). Temporal dynamics of lung aeration determined by dynamic CT in a porcine model of ARDS. *British Journal of Anaesthesia* **87**(3): p. 459-68.

96. Markstaller, K., Kauczor, H.U., Weiler, N., Karmrodt, J., Doebrich, M., Ferrante, M., Thelen, M., and Eberle, B. (**2003**). Lung density distribution in dynamic CT correlates with oxygenation in ventilated pigs with lavage ARDS. *British Journal of Anaesthesia* **91**(5): p. 699-708.

97. Neumann, P., Berglund, J.E., Fernandez Mondejar, E., Magnusson, A., and Hedenstierna, G. (**1998**). Dynamics of lung collapse and recruitment during prolonged breathing in porcine lung injury. *Journal of Applied Physiology* **85**(4): p. 1533-43.

98. Halter, J.M., Steinberg, J.M., Schiller, H.J., DaSilva, M., Gatto, L.A., Landas, S., and Nieman, G.F. (**2003**). Positive end-expiratory pressure after a recruitment maneuver prevents both alveolar collapse and recruitment/derecruitment. *American Journal of Respiratory and Critical Care Medicine* **167**(12): p. 1620-6.

99. McCann, U.G., 2nd, Schiller, H.J., Carney, D.E., Gatto, L.A., Steinberg, J.M., and Nieman, G.F. (**2001**). Visual validation of the mechanical stabilizing effects of positive end-expiratory pressure at the alveolar level. *The Journal of Surgical Research* **99**(2): p. 335-42.

100. Schiller, H.J., McCann, U.G., 2nd, Carney, D.E., Gatto, L.A., Steinberg, J.M., and Nieman, G.F. (**2001**). Altered alveolar mechanics in the acutely injured lung. *Critical Care Medicine* **29**(5): p. 1049-55.

101. Baumgardner, J.E., Otto, C.M., and Markstaller, K. (**2013**). Cyclic recruitment of atelectasis – Are there implications for our clinical practice? *Trends in Anaesthesia and Critical Care* **3**(4): p. 205-210.

102. Bates, J.H. and Irvin, C.G. (**2002**). Time dependence of recruitment and derecruitment in the lung: a theoretical model. *Journal of Applied Physiology* **93**(2): p. 705-13.

103. Bilek, A.M., Dee, K.C., and Gaver, D.P. (**2003**). Mechanisms of surface-tension-induced epithelial cell damage in a model of pulmonary airway reopening. *Journal of Applied Physiology* **94**(2): p. 770-783.

104. Tschumperlin, D.J., Oswari, J., and Margulies, A.S. (**2000**). Deformation-induced injury of alveolar epithelial cells. Effect of frequency, duration, and amplitude. *American Journal of Respiratory and Critical Care Medicine* **162**(2 Pt 1): p. 357-62.

105. Davidovich, N., DiPaolo, B.C., Lawrence, G.G., Chhour, P., Yehya, N., and Margulies, S.S. (**2013**). Cyclic stretch-induced oxidative stress increases pulmonary alveolar epithelial permeability. *American Journal of Respiratory Cell and Molecular Biology* **49**(1): p. 156-64.

106. Chapman, K.E., Sinclair, S.E., Zhuang, D., Hassid, A., Desai, L.P., and Waters, C.M. (**2005**). Cyclic mechanical strain increases reactive oxygen species production in pulmonary epithelial cells. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **289**(5): p. L834-41.

107. Wellman, T.J., de Prost, N., Tucci, M., Winkler, T., Baron, R.M., Filipczak, P., Raby, B., Chu, J.H., Harris, R.S., Musch, G., Dos Reis Falcao, L.F., Capelozzi, V., Venegas, J.G., and Vidal Melo, M.F. (**2016**). Lung Metabolic Activation as an Early Biomarker of Acute Respiratory Distress Syndrome and Local Gene Expression Heterogeneity. *Anesthesiology* **125**(5): p. 992-1004.

108. Wellman, T.J., Winkler, T., Costa, E.L., Musch, G., Harris, R.S., Venegas, J.G., and Vidal Melo, M.F. (**2012**). Effect of regional lung inflation on ventilation heterogeneity at different length scales during mechanical ventilation of normal sheep lungs. *J Appl Physiol (1985)* **113**(6): p. 947-57.

109. Lachmann, B. (**1992**). Open up the lung and keep the lung open. *Intensive Care Medicine* **18**(6): p. 319-21.

110. Bone, R.C. (**1996**). Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation. *Critical Care Medicine* **24**(1): p. 163-72.

111. Borrelli, E., Roux-Lombard, P., Grau, G.E., Girardin, E., Ricou, B., Dayer, J., and Suter, P.M. (**1996**). Plasma concentrations of cytokines, their soluble receptors, and antioxidant vitamins can predict the development of multiple organ failure in patients at risk. *Critical Care Medicine* **24**(3): p. 392-7.

112. Parker, T.G. (**1995**). Molecular biology of myocardial hypertrophy and failure: gene expression and trophic signaling. *New Horizons* **3**(2): p. 288-300.

113. Slutsky, A.S. and Tremblay, L.N. (**1998**). Multiple system organ failure: is mechanical ventilation a contributing factor? *American Journal of Respiratory and Critical Care Medicine* **157**(6): p. 1721-1725.

114. Tremblay, L. and Slutsky, A.S. (**1998**). Ventilator-induced injury: from barotrauma to biotrauma. *Proceedings of the Association of American Physicians* **110**(6): p. 482-488.

115. Imai, Y., Kawano, T., Miyasaka, K., Takata, M., Imai, T., and Okuyama, K. (**1994**). Inflammatory chemical mediators during conventional ventilation and during high frequency oscillatory ventilation. *American Journal of Respiratory and Critical Care Medicine* **150**(6 Pt 1): p. 1550-4.

116. Kawano, T., Mori, S., Cybulsky, M., Burger, R., Ballin, A., Cutz, E., and Bryan, A.C. (**1987**). Effect of granulocyte depletion in a ventilated surfactant-depleted lung. *Journal of Applied Physiology* **62**(1): p. 27-33.

117. Sugiura, M., McCulloch, P.R., Wren, S., Dawson, R.H., and Froese, A.B. (**1994**). Ventilator pattern influences neutrophil influx and activation in atelectasis-prone rabbit lung. *Journal of Applied Physiology* **77**(3): p. 1355-65.

118. Tremblay, L., Valenza, F., Ribeiro, S.P., Li, J., and Slutsky, A.S. (**1997**). Injurious ventilatory strategies increase cytokines and c-fos m-RNA expression in an isolated rat lung model. *The Journal of Clinical Investigation* **99**(5): p. 944-952.

119. Vlahakis, N.E., Schroeder, M.A., Limper, A.H., and Hubmayr, R.D. (**1999**). Stretch induces cytokine release by alveolar epithelial cells in vitro. *The American Journal of Physiology* **277**(1): p. L167-73.

120. Enhorning, G. (**2008**). Surfactant in airway disease. *Chest* **133**(4): p. 975-80.

121. Kerr, M.H. and Paton, J.Y. (**1999**). Surfactant protein levels in severe respiratory syncytial virus infection. *American Journal of Respiratory and Critical Care Medicine* **159**(4 Pt 1): p. 1115-8.

122. Clark, J.C., Wert, S.E., Bachurski, C.J., Stahlman, M.T., Stripp, B.R., Weaver, T.E., and Whitsett, J.A. (**1995**). Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proceedings of the National Academy of Sciences of the United States of America* **92**(17): p. 7794-8.

123. Ingenito, E.P., Mora, R., Cullivan, M., Marzan, Y., Haley, K., Mark, L., and Sonna, L.A. (**2001**). Decreased surfactant protein-B expression and surfactant dysfunction in a murine model of acute lung injury. *American Journal of Respiratory Cell and Molecular Biology* **25**(1): p. 35-44.

124. Luchetti, M., Ferrero, F., Gallini, C., Natale, A., Pigna, A., Tortorolo, L., and Marraro, G. (**2002**). Multicenter, randomized, controlled study of porcine surfactant in severe respiratory syncytial virus-induced respiratory failure. *Pediatric Critical Care Medicine* **3**(3): p. 261-268.

125. Tibby, S.M., Hatherill, M., Wright, S.M., Wilson, P., Postle, A.D., and Murdoch, I.A. (**2000**). Exogenous surfactant supplementation in infants with respiratory syncytial virus bronchiolitis. *American Journal of Respiratory and Critical Care Medicine* **162**(4 Pt 1): p. 1251-6.

126. Thammanomai, A., Hamakawa, H., Bartolak-Suki, E., and Suki, B. (**2013**). Combined effects of ventilation mode and positive end-expiratory pressure on mechanics, gas exchange and the epithelium in mice with acute lung injury. *PLoS One* **8**(1): p. e53934.

127. Jaecklin, T., Jarreau, P.-H., and Kavanagh, B.P. (**2015**). Ventilator-associated lung injury, in *Pediatric and Neonatal Mechanical Ventilation*. Springer. p. 917-945.

128. Uhlig, U., Haitsma, J.J., Goldmann, T., Poelma, D.L., Lachmann, B., and Uhlig, S. (**2002**). Ventilation-induced activation of the mitogen-activated protein kinase pathway. *The European Respiratory Journal* **20**(4): p. 946-56.

129. Fischer, O.M., Hart, S., Gschwind, A., and Ullrich, A. (**2003**). EGFR signal transactivation in cancer cells. *Biochemical Society Transactions* **31**(Pt 6): p. 1203-8.

130. Bierman, A., Yerrapureddy, A., Reddy, N.M., Hassoun, P.M., and Reddy, S.P. (**2008**). Epidermal growth factor receptor (EGFR) regulates mechanical ventilation-induced lung injury in mice. *Translational Research: The Journal of Laboratory and Clinical Medicine* **152**(6): p. 265-72.

131. Correa-Meyer, E., Pesce, L., Guerrero, C., and Sznajder, J.I. (**2002**). Cyclic stretch activates ERK1/2 via G proteins and EGFR in alveolar epithelial cells. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **282**(5): p. L883-91.

132. Tschumperlin, D.J., Shively, J.D., Swartz, M.A., Silverman, E.S., Haley, K.J., Raab, G., and Drazen, J.M. (**2002**). Bronchial epithelial compression regulates MAP kinase signaling and HB-EGF-like growth factor expression. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **282**(5): p. L904-11.

133. Zaher, T.E., Miller, E.J., Morrow, D.M., Javdan, M., and Mantell, L.L. (**2007**). Hyperoxia-induced signal transduction pathways in pulmonary epithelial cells. *Free Radical Biology and Medicine* **42**(7): p. 897-908.

134. Guijarro-Munoz, I., Compte, M., Alvarez-Cienfuegos, A., Alvarez-Vallina, L., and Sanz, L. (**2014**). Lipopolysaccharide activates toll-like receptor 4 (TLR4)-mediated NF-kappaB signaling pathway and proinflammatory response in human pericytes. *The Jounal of Biological Chemistry* **289**(4): p. 2457-68.

135. Cho, H.Y., Jedlicka, A.E., Reddy, S.P., Kensler, T.W., Yamamoto, M., Zhang, L.Y., and Kleeberger, S.R. (**2002**). Role of NRF2 in protection against hyperoxic lung injury in mice. *American Journal of Respiratory Cell and Molecular Biology* **26**(2): p. 175-82.

136. Shan, Y., Akram, A., Amatullah, H., Zhou, D.Y., Gali, P.L., Maron-Gutierrez, T., Gonzalez-Lopez, A., Zhou, L., Rocco, P.R., Hwang, D., Albaiceta, G.M., Haitsma, J.J., and dos Santos, C.C. (**2015**). ATF3 protects pulmonary resident cells from acute and ventilator-induced lung injury by preventing Nrf2 degradation. *Antioxidants and Redox Signaling* **22**(8): p. 651-68.

137. Marzec, J.M., Christie, J.D., Reddy, S.P., Jedlicka, A.E., Vuong, H., Lanken, P.N., Aplenc, R., Yamamoto, T., Yamamoto, M., Cho, H.Y., and Kleeberger, S.R. (**2007**). Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. *Faseb Journal* **21**(9): p. 2237-46.

138. Meduri, G.U., Headley, S., Kohler, G., Stentz, F., Tolley, E., Umberger, R., and Leeper, K. (**1995**). Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome over time. *Chest* **107**(4): p. 1062-73.

139. Ranieri, V., Suter, P.M., Tortorella, C., and et al. (**1999**). Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: A randomized controlled trial. *Journal of the American Medical Association* **282**(1): p. 54-61.

140. Stuber, F., Wrigge, H., Schroeder, S., Wetegrove, S., Zinserling, J., Hoeft, A., and Putensen, C. (**2002**). Kinetic and reversibility of mechanical ventilation-associated pulmonary and systemic inflammatory response in patients with acute lung injury. *Intensive Care Medicine* **28**(7): p. 834-41.

141. Yoshikawa, S., King, J.A., Lausch, R.N., Penton, A.M., Eyal, F.G., and Parker, J.C. (**2004**). Acute ventilator-induced vascular permeability and cytokine responses in isolated and in situ mouse lungs. *Journal of Applied Physiology* **97**(6): p. 2190-9.

142. Pugin, J. (**2003**). Molecular mechanisms of lung cell activation induced by cyclic stretch. *Critical Care Medicine* **31**(4 Suppl): p. S200-6.

143. Waragai, A., Yamashita, H., Hosoi, K., Hoshina, H., Noda, E., Yan, K., and Kawano, T. (**2007**). High-frequency oscillation (HFO) prevents activation of NF-kappaB found with conventional mechanical ventilation (CMV) in surfactant-depleted rabbit lung. *Pediatric Pulmonology* **42**(5): p. 440-5.

144. Chiang, C.H., Pai, H.I., and Liu, S.L. (**2008**). Ventilator-induced lung injury (VILI) promotes ischemia/reperfusion lung injury (I/R) and NF-kappaB antibody attenuates both injuries. *Resuscitation* **79**(1): p. 147-54.

145. van Kooten, C., van der Linde, X., Woltman, A.M., van Es, L.A., and Daha, M.R. (**1999**). Synergistic effect of interleukin-1 and CD40L on the activation of human renal tubular epithelial cells. *Kidney International* **56**(1): p. 41-51.

146. Prodjosudjadi, W., Gerritsma, J.S., Klar-Mohamad, N., Gerritsen, A.F., Bruijn, J.A., Daha, M.R., and van Es, L.A. (**1995**). Production and cytokine-mediated regulation of monocyte chemoattractant protein-1 by human proximal tubular epithelial cells. *Kidney International* **48**(5): p. 1477-86.

147. Biswas, P., Delfanti, F., Bernasconi, S., Mengozzi, M., Cota, M., Polentarutti, N., Mantovani, A., Lazzarin, A., Sozzani, S., and Poli, G. (**1998**). Interleukin-

6 induces monocyte chemotactic protein-1 in peripheral blood mononuclear cells and in the U937 cell line. *Blood* **91**(1): p. 258-65.

148. Altemeier, W.A., Matute-Bello, G., Frevert, C.W., Kawata, Y., Kajikawa, O., Martin, T.R., and Glenny, R.W. (**2004**). Mechanical ventilation with moderate tidal volumes synergistically increases lung cytokine response to systemic endotoxin. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **287**(3): p. L533-L542.

149. Schmidt, A.M., Yan, S.D., Yan, S.F., and Stern, D.M. (**2001**). The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *The Journal of Clinical Investigation* **108**(7): p. 949-55.

150. Uchida, T., Shirasawa, M., Ware, L.B., Kojima, K., Hata, Y., Makita, K., Mednick, G., Matthay, Z.A., and Matthay, M.A. (**2006**). Receptor for advanced glycation end-products is a marker of type I cell injury in acute lung injury. *American Journal of Respiratory and Critical Care Medicine* **173**(9): p. 1008-15.

151. Calfee, C.S., Ware, L.B., Eisner, M.D., Parsons, P.E., Thompson, B.T., Wickersham, N., and Matthay, M.A. (**2008**). Plasma receptor for advanced glycation end products and clinical outcomes in acute lung injury. *Thorax* **63**(12): p. 1083-9.

152. Griffiths, M.J. and McAuley, D.F. (**2008**). RAGE: a biomarker for acute lung injury. *Thorax* **63**(12): p. 1034-6.

153. Ong, T., McClintock, D.E., Kallet, R.H., Ware, L.B., Matthay, M.A., and Liu, K.D. (**2010**). Ratio of angiopoietin-2 to angiopoietin-1 as a predictor of mortality in acute lung injury patients. *Critical Care Medicine* **38**(9): p. 1845-51.

154. van der Heijden, M., van Nieuw Amerongen, G.P., Koolwijk, P., van Hinsbergh, V.W., and Groeneveld, A.B. (**2008**). Angiopoietin-2, permeability oedema, occurrence and severity of ALI/ARDS in septic and non-septic critically ill patients. *Thorax* **63**(10): p. 903-9.

155. Zinter, M.S., Spicer, A., Orwoll, B.O., Alkhouli, M., Dvorak, C.C., Calfee, C.S., Matthay, M.A., and Sapru, A. (**2016**). Plasma angiopoietin-2 outperforms other markers of endothelial injury in prognosticating pediatric ARDS mortality. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **310**(3): p. L224-31.

156. Tsangaris, I., Tsantes, A., Vrigkou, E., Kopterides, P., Pelekanou, A., Zerva, K., Antonakos, G., Konstantonis, D., Mavrou, I., Tsaknis, G., Kyriazopoulou, E., Mouktaroudi, M., Kokori, S., Orfanos, S.E., Giamarellos-Bourboulis, E.J., and Armaganidis, A. (**2017**). Angiopoietin-2 levels as predictors of outcome in mechanically ventilated patients with acute respiratory distress syndrome. *Disease Markers* **2017**: p. 6758721.

157. Cenani, A., Cerri, S., Gougnard, A., Detilleux, J., Franck, T., Serteyn, D., and Sandersen, C. (**2014**). Effect of high pressure-volume and low pressure-volume mechanical ventilation on plasma concentrations of inflammatory markers in horses during general anesthesia. *Journal of Anesthesia and Clinical Research* **5**.

158. Borregaard, N. and Cowland, J.B. (**1997**). Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**(10): p. 3503-21.

159. Albaiceta, G.M., Gutierrez-Fernandez, A., Parra, D., Astudillo, A., Garcia-Prieto, E., Taboada, F., and Fueyo, A. (**2008**). Lack of matrix metalloproteinase-9 worsens ventilator-induced lung injury. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **294**(3): p. L535-43.

160. Bregeon, F., Steinberg, J.G., Andreotti, N., Sabatier, J.M., Delpierre, S., Ravailhe, S., and Jammes, Y. (**2010**). Substance P receptor blockade decreases stretch-induced lung cytokines and lung injury in rats. *The Journal of Physiology* **588**(Pt 8): p. 1309-19.

161. Levi, M., van der Poll, T., and Buller, H.R. (**2004**). Bidirectional relation between inflammation and coagulation. *Circulation* **109**(22): p. 2698-704.

162. Gunther, A., Mosavi, P., Heinemann, S., Ruppert, C., Muth, H., Markart, P., Grimminger, F., Walmrath, D., Temmesfeld-Wollbruck, B., and Seeger, W. (**2000**). Alveolar fibrin formation caused by enhanced procoagulant and depressed fibrinolytic capacities in severe pneumonia. Comparison with the acute respiratory distress syndrome. *American Journal of Respiratory and Critical Care Medicine* **161**(2 Pt 1): p. 454-62.

163. Schultz, M.J., Millo, J., Levi, M., Hack, C.E., Weverling, G.J., Garrard, C.S., and van der Poll, T. (**2004**). Local activation of coagulation and inhibition of fibrinolysis in the lung during ventilator associated pneumonia. *Thorax* **59**(2): p. 130-5.

164. Camprubi-Rimblas, M., Tantinya, N., Bringue, J., Guillamat-Prats, R., and Artigas, A. (**2018**). Anticoagulant therapy in acute respiratory distress syndrome. *Annals of Translational Medicine* **6**(2): p. 36.

165. Prabhakaran, P., Ware, L.B., White, K.E., Cross, M.T., Matthay, M.A., and Olman, M.A. (**2003**). Elevated levels of plasminogen activator inhibitor-1 in pulmonary edema fluid are associated with mortality in acute lung injury. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **285**(1): p. L20-L28.

166. Chen, C.M., Chou, H.C., Wang, L.F., and Lang, Y.D. (**2008**). Captopril decreases plasminogen activator inhibitor-1 in rats with ventilator-induced lung injury. *Critical Care Medicine* **36**(6): p. 1880-5.

167. Heise, R.L., Stober, V., Cheluvaraju, C., Hollingsworth, J.W., and Garantziotis, S. (**2011**). Mechanical stretch induces epithelial-mesenchymal transition in alveolar epithelia via hyaluronan activation of innate immunity. *The Journal of Biological Chemistry* **286**(20): p. 17435-44.

168. Cabrera-Benitez, N.E., Laffey, J.G., Parotto, M., Spieth, P.M., Villar, J., Zhang, H., and Slutsky, A.S. (**2014**). Mechanical ventilation-associated lung fibrosis in acute respiratory distress syndrome: a significant contributor to poor outcome. *Anesthesiology* **121**(1): p. 189-98.

169. Cabrera-Benitez, N.E., Parotto, M., Post, M., Han, B., Spieth, P.M., Cheng, W.E., Valladares, F., Villar, J., Liu, M., Sato, M., Zhang, H., and Slutsky, A.S. (**2012**). Mechanical stress induces lung fibrosis by epithelial-mesenchymal transition. *Critical Care Medicine* **40**(2): p. 510-7.

170. Suki, B. and Hubmayr, R. (**2014**). Epithelial and endothelial damage induced by mechanical ventilation modes. *Current Opinion in Critical Care* **20**(1): p. 17-24.

171. Gu, C., Liu, M., Zhao, T., Wang, D., and Wang, Y. (**2015**). Protective role of p120-catenin in maintaining the integrity of adherens and tight junctions in ventilator-induced lung injury. *Respiratory Research* **16**: p. 58.

172. Wang, Y., Minshall, R.D., Schwartz, D.E., and Hu, G. (**2011**). Cyclic stretch induces alveolar epithelial barrier dysfunction via calpain-mediated degradation of p120-catenin. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **301**(2): p. L197-206.

173. Villar, J., Cabrera, N.E., Valladares, F., Casula, M., Flores, C., Blanch, L., Quilez, M.E., Santana-Rodriguez, N., Kacmarek, R.M., and Slutsky, A.S. (**2011**). Activation of the Wnt/beta-catenin signaling pathway by mechanical ventilation is associated with ventilator-induced pulmonary fibrosis in healthy lungs. *PLoS One* **6**(9): p. e23914.

174. Zupancich, E., Paparella, D., Turani, F., Munch, C., Rossi, A., Massaccesi, S., and Ranieri, V.M. (**2005**). Mechanical ventilation affects inflammatory mediators in patients undergoing cardiopulmonary bypass for cardiac surgery: a randomized clinical trial. *The Journal of Thoracic and Cardiovascular Surgery* **130**(2): p. 378-83.

175. Wrigge, H., Uhlig, U., Baumgarten, G., Menzenbach, J., Zinserling, J., Ernst, M., Dromann, D., Welz, A., Uhlig, S., and Putensen, C. (**2005**). Mechanical ventilation strategies and inflammatory responses to cardiac surgery: a prospective randomized clinical trial. *Intensive Care Medicine* **31**(10): p. 1379-87.

176. Koner, O., Celebi, S., Balci, H., Cetin, G., Karaoglu, K., and Cakar, N. (**2004**). Effects of protective and conventional mechanical ventilation on pulmonary function and systemic cytokine release after cardiopulmonary bypass. *Intensive Care Medicine* **30**(4): p. 620-6.

177. Wolthuis, E.K., Choi, G., Dessing, M.C., Bresser, P., Lutter, R., Dzoljic, M., van der Poll, T., Vroom, M.B., Hollmann, M., and Schultz, M.J. (**2008**). Mechanical ventilation with lower tidal volumes and positive end-expiratory pressure prevents pulmonary inflammation in patients without preexisting lung injury. *Anesthesiology* **108**(1): p. 46-54.

178. Wrigge, H., Uhlig, U., Zinserling, J., Behrends-Callsen, E., Ottersbach, G., Fischer, M., Uhlig, S., and Putensen, C. (**2004**). The effects of different

ventilatory settings on pulmonary and systemic inflammatory responses during major surgery. *Anesthesia and Analgesia* **98**(3): p. 775-81.

179. Wrigge, H., Zinserling, J., Stuber, F., von Spiegel, T., Hering, R., Wetegrove, S., Hoeft, A., and Putensen, C. (**2000**). Effects of mechanical ventilation on release of cytokines into systemic circulation in patients with normal pulmonary function. *Anesthesiology* **93**(6): p. 1413-7.

180. Tremblay, L.N., Miatto, D., Hamid, Q., Govindarajan, A., and Slutsky, A.S. (**2002**). Injurious ventilation induces widespread pulmonary epithelial expression of tumor necrosis factor-alpha and interleukin-6 messenger RNA. *Critical Care Medicine* **30**(8): p. 1693-700.

181. Chu, E.K., Whitehead, T., and Slutsky, A.S. (**2004**). Effects of cyclic opening and closing at low- and high-volume ventilation on bronchoalveolar lavage cytokines. *Critical Care Medicine* **32**(1): p. 168-74.

182. Bouadma, L., Dreyfuss, D., Ricard, J.-D., Martet, G., and Saumon, G. (**2007**). Mechanical ventilation and hemorrhagic shock-resuscitation interact to increase inflammatory cytokine release in rats. *Critical Care Medicine* **35**(11): p. 2601-2606.

183. Hong, C.M., Xu, D.Z., Lu, Q., Cheng, Y., Pisarenko, V., Doucet, D., Brown, M., Aisner, S., Zhang, C., Deitch, E.A., and Delphin, E. (**2010**). Low tidal volume and high positive end-expiratory pressure mechanical ventilation results in increased inflammation and ventilator-associated lung injury in normal lungs. *Anesthesia and Analgesia* **110**(6): p. 1652-60.

184. Cannizzaro, V., Hantos, Z., Sly, P.D., and Zosky, G.R. (**2011**). Linking lung function and inflammatory responses in ventilator-induced lung injury. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **300**(1): p. L112-L120.

185. Chiumello, D., Pristine, G., and Slutsky, A.S. (**1999**). Mechanical ventilation affects local and systemic cytokines in an animal model of acute respiratory distress syndrome. *American Journal of Respiratory and Critical Care Medicine* **160**(1): p. 109-16.

186. Slutsky, A.S. (**2015**). History of mechanical ventilation. From Vesalius to ventilator-induced lung injury. *American Journal of Respiratory and Critical Care Medicine* **191**(10): p. 1106-1115.

187. Carrasco Loza, R., Villamizar Rodriguez, G., and Medel Fernandez, N. (**2015**). Ventilator-induced lung injury (VILI) in acute respiratory distress syndrome (ARDS): Volutrauma and molecular effects. *The Open Respiratory Medicine Journal* **9**: p. 112-9.

188. Bailey, T.C., Maruscak, A.A., Martin, E.L., Forbes, A.R., Petersen, A., McCaig, L.A., Yao, L.-J., Lewis, J.F., and Veldhuizen, R.A.W. (**2008**). The effects of long-term conventional mechanical ventilation on the lungs of adult rats. *Critical Care Medicine* **36**(8): p. 2381-2387.

189. Guerin, C., Reignier, J., Richard, J.C., Beuret, P., Gacouin, A., Boulain, T., Mercier, E., Badet, M., Mercat, A., Baudin, O., Clavel, M., Chatellier, D., Jaber, S., Rosselli, S., Mancebo, J., Sirodot, M., Hilbert, G., Bengler, C., Richecoeur, J., Gainnier, M., Bayle, F., Bourdin, G., Leray, V., Girard, R., Baboi, L., and Ayzac, L. (**2013**). Prone positioning in severe acute respiratory distress syndrome. *The New England Journal of Medicine* **368**(23): p. 2159-68.

190. Cavalcanti, A.B., Suzumura, E.A., Laranjeira, L.N., Paisani, D.M., Damiani, L.P., Guimaraes, H.P., Romano, E.R., Regenga, M.M., Taniguchi, L.N.T., Teixeira, C., Pinheiro de Oliveira, R., Machado, F.R., Diaz-Quijano, F.A., Filho, M.S.A., Maia, I.S., Caser, E.B., Filho, W.O., Borges, M.C., Martins,

P.A., Matsui, M., Ospina-Tascon, G.A., Giancursi, T.S., Giraldo-Ramirez, N.D., Vieira, S.R.R., Assef, M., Hasan, M.S., Szczeklik, W., Rios, F., Amato, M.B.P., Berwanger, O., and Ribeiro de Carvalho, C.R. (**2017**). Effect of lung recruitment and titrated positive end-expiratory pressure (PEEP) vs low PEEP on mortality in patients with acute respiratory distress syndrome: A randomized clinical trial. *Journal of the American Medical Association* **318**(14): p. 1335-1345.

191. Wellman, T.J., Winkler, T., Costa, E.L., Musch, G., Harris, R.S., Zheng, H., Venegas, J.G., and Vidal Melo, M.F. (**2014**). Effect of local tidal lung strain on inflammation in normal and lipopolysaccharide-exposed sheep. *Critical Care Medicine* **42**(7): p. e491-500.

192. Dubsky, S., Hooper, S.B., Siu, K.K.W., and Fouras, A. (**2012**). Synchrotron-based dynamic computed tomography of tissue motion for regional lung function measurement. *Journal of The Royal Society Interface* **9**(74): p. 2213-2224.

193. Larsson, D.H., Takman, P.A., Lundstrom, U., Burvall, A., and Hertz, H.M. (**2011**). A 24 keV liquid-metal-jet x-ray source for biomedical applications. *The Review of Scientific Instruments* **82**(12): p. 123701.

194. Dubsky, S., Zosky, G.R., Perks, K., Samarage, C.R., Henon, Y., Hooper, S.B., and Fouras, A. (**2017**). Assessment of airway response distribution and paradoxical airway dilation in mice during methacholine challenge. *Journal of Applied Physiology* **122**(3): p. 503-510.

195. Kim, E.H., Preissner, M., Carnibella, R.P., Samarage, C.R., Bennett, E., Diniz, M.A., Fouras, A., Zosky, G.R., and Jones, H.D. (**2017**). Novel analysis of 4DCT imaging quantifies progressive increases in anatomic dead space during

mechanical ventilation in mice. *Journal of Applied Physiology* **123**(3): p. 578-584.

196. Stahr, C.S., Samarage, C.R., Donnelley, M., Farrow, N., Morgan, K.S., Zosky, G., Boucher, R.C., Siu, K.K., Mall, M.A., Parsons, D.W., Dubsky, S., and Fouras, A. (**2016**). Quantification of heterogeneity in lung disease with image-based pulmonary function testing. *Scientific Reports* **6**.

197. Frangi, A.F., Niessen, W.J., Vincken, K.L., and Viergever, M.A. (**1998**). Multiscale vessel enhancement filtering, in *Medical Image Computing and Computer-Assisted Intervention — MICCAI'98: First International Conference Cambridge, MA, USA, October 11–13, 1998*, Wells, W.M., Colchester, A., and Delp, S., Editors. Springer Berlin Heidelberg: Berlin, Heidelberg. p. 130-137.

198. Livak, K.J. and Schmittgen, T.D. (**2001**). Analysis of relative gene expression data using Real-Time quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**(4): p. 402-408.

199. Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X.F., and Achong, M.K. (**1998**). IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *The Journal of Clinical Investigation* **101**(2): p. 311-20.

200. Hosaka, K., Rojas, K., Fazal, H.Z., Schneider, M.B., Shores, J., Federico, V., McCord, M., Lin, L., and Hoh, B. (**2017**). Monocyte chemotactic protein-1-Interleukin-6-Osteopontin pathway of intra-aneurysmal tissue healing. *Stroke* **48**(4): p. 1052-1060.

201. Viedt, C., Dechend, R., Fei, J., Hansch, G.M., Kreuzer, J., and Orth, S.R. (**2002**). MCP-1 induces inflammatory activation of human tubular epithelial cells: involvement of the transcription factors, nuclear factor-kappaB and

activating protein-1. *Journal of the American Society of Nephrology* **13**(6): p. 1534-47.

202. Tesch, G.H., Schwarting, A., Kinoshita, K., Lan, H.Y., Rollins, B.J., and Kelley, V.R. (**1999**). Monocyte chemoattractant protein-1 promotes macrophage-mediated tubular injury, but not glomerular injury, in nephrotoxic serum nephritis. *The Journal of Clinical Investigation* **103**(1): p. 73-80.

203. Shosholcheva, M., capital Je, S.N., Kartalov, A., Kuzmanovska, B., and Miladinova, D. (**2017**). Synergistic effect of hyperoxia and biotrauma on ventilator-induced lung injury. *Prilozi. Contributions* **38**(1): p. 91-96.

204. Silva, P.L., Negrini, D., and Macêdo Rocco, P.R. (**2015**). Mechanisms of ventilator-induced lung injury in healthy lungs. *Best Practice and Research Clinical Anaesthesiology* **29**(3): p. 301-313.

205. De Filippo, K., Dudeck, A., Hasenberg, M., Nye, E., van Rooijen, N., Hartmann, K., Gunzer, M., Roers, A., and Hogg, N. (**2013**). Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. *Blood* **121**(24): p. 4930-7.

206. Ozaki, T. and Nakagawara, A. (**2011**). Role of p53 in cell death and human cancers. *Cancers (Basel)* **3**(1): p. 994-1013.

207. Tang, X., Asano, M., O'Reilly, A., Farquhar, A., Yang, Y., and Amar, S. (**2012**). p53 is an important regulator of CCL2 gene expression. *Current Molecular Medicine* **12**(8): p. 929-43.

208. Wolters, P.J., Wray, C., Sutherland, R.E., Kim, S.S., Koff, J., Mao, Y., and Frank, J.A. (**2009**). Neutrophil-derived IL-6 limits alveolar barrier disruption in experimental ventilator-induced lung injury. *Journal of Immunology* **182**(12): p. 8056-62.

209. Bhandari, V., Choo-Wing, R., Lee, C.G., Zhu, Z., Nedrelow, J.H., Chupp, G.L., Zhang, X., Matthay, M.A., Ware, L.B., Homer, R.J., Lee, P.J., Geick, A., de Fougerolles, A.R., and Elias, J.A. (**2006**). Hyperoxia causes angiopoietin 2-mediated acute lung injury and necrotic cell death. *Nature Medicine* **12**(11): p. 1286-93.

210. Yen, S., Preissner, M., Bennett, E., Dubsky, S., Carnibella, R., O'Toole, R., Roddam, L., Jones, H., Dargaville, P.A., Fouras, A., and Zosky, G.R. (**2019**). The link between regional tidal stretch and lung injury during mechanical ventilation. *American Journal of Respiratory Cell and Molecular Biology* **60**(5): p. 569-577.

211. Preissner, M., Murrie, R.P., Pinar, I., Werdiger, F., Carnibella, R.P., Zosky, G.R., Fouras, A., and Dubsky, S. (**2018**). High resolution propagation-based imaging system for in vivo dynamic computed tomography of lungs in small animals. *Phys Med Biol* **63**(8): p. 08nt03.

212. Zosky, G.R., Janosi, T.Z., Adamicza, Á., Bozanich, E.M., Cannizzaro, V., Larcombe, A.N., Turner, D.J., Sly, P.D., and Hantos, Z. (**2008**). The bimodal quasi-static and dynamic elastance of the murine lung. *Journal of Applied Physiology* **105**(2): p. 685-692.

213. Sinclair, S.E., Molthen, R.C., Haworth, S.T., Dawson, C.A., and Waters, C.M. (**2007**). Airway strain during mechanical ventilation in an intact animal model. *American Journal of Respiratory and Critical Care Medicine* **176**(8): p. 786-794.

214. Amato, M.B., Meade, M.O., Slutsky, A.S., Brochard, L., Costa, E.L., Schoenfeld, D.A., Stewart, T.E., Briel, M., Talmor, D., Mercat, A., Richard, J.C., Carvalho, C.R., and Brower, R.G. (**2015**). Driving pressure and survival

in the acute respiratory distress syndrome. *The New England Journal of Medicine* **372**(8): p. 747-55.

215. Naik, A.S., Kallapur, S.G., Bachurski, C.J., Jobe, A.H., Michna, J., Kramer, B.W., and Ikegami, M. (**2001**). Effects of ventilation with different positive end-expiratory pressures on cytokine expression in the preterm lamb lung. *American Journal of Respiratory and Critical Care Medicine* **164**(3): p. 494-8.

216. Kozue, K., Inaoka, H., Kokubo, K., and Kobayashi, H. (**2018**). Effects of positive end-expiratory pressure-like cyclic stretching on the production of interleukin-6 protein in normal human pulmonary artery endothelial cells. *European Respiratory Journal* **52**(suppl 62): p. PA4306.

217. DiPaolo, B.C., Lenormand, G., Fredberg, J.J., and Margulies, S.S. (**2010**). Stretch magnitude and frequency-dependent actin cytoskeleton remodeling in alveolar epithelia. *American Journal of Physiology. Cell Physiology* **299**(2): p. C345-53.

218. dos Santos, C.C., Han, B., Andrade, C.F., Bai, X., Uhlig, S., Hubmayr, R., Tsang, M., Lodyga, M., Keshavjee, S., Slutsky, A.S., and Liu, M. (**2004**). DNA microarray analysis of gene expression in alveolar epithelial cells in response to TNFalpha, LPS, and cyclic stretch. *Physiol Genomics* **19**(3): p. 331-42.

219. Nieto, J.E., Macdonald, M.H., Braim, A.E.P., and Aleman, M. (**2009**). Effect of lipopolysaccharide infusion on gene expression of inflammatory cytokines in normal horses in vivo. *Equine Veterinary Journal* **41**(7): p. 717-719.

220. Ding, N., Wang, F., Xiao, H., Xu, L., and She, S. (**2013**). Mechanical ventilation enhances HMGB1 expression in an LPS-induced lung injury model. *PLoS One* **8**(9): p. e74633.

221. O'Mahony, D.S., Liles, W.C., Altemeier, W.A., Dhanireddy, S., Frevert, C.W., Liggitt, D., Martin, T.R., and Matute-Bello, G. (**2006**). Mechanical ventilation interacts with endotoxemia to induce extrapulmonary organ dysfunction. *Critical Care* **10**(5): p. R136-R136.

222. Okutani, D., Han, B., Mura, M., Waddell, T.K., Keshavjee, S., and Liu, M. (**2007**). High-volume ventilation induces pentraxin 3 expression in multiple acute lung injury models in rats. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **292**(1): p. L144-L153.

223. Haslett, C., Worthen, G.S., Giclas, P.C., Morrison, D.C., Henson, J.E., and Henson, P.M. (**1987**). The pulmonary vascular sequestration of neutrophils in endotoxemia is initiated by an effect of endotoxin on the neutrophil in the rabbit. *The American Review of Respiratory Disease* **136**(1): p. 9-18.

224. Uchiba, M., Okajima, K., Murakami, K., Okabe, H., and Takatsuki, K. (**1995**). Endotoxin-induced pulmonary vascular injury is mainly mediated by activated neutrophils in rats. *Thrombosis Research* **78**(2): p. 117-25.

225. Mullen, P.G., Windsor, A.C., Walsh, C.J., Fowler, A.A., 3rd, and Sugerman, H.J. (**1995**). Tumor necrosis factor-alpha and interleukin-6 selectively regulate neutrophil function in vitro. *The Journal of Surgical Research* **58**(2): p. 124-30.

226. Bank, U., Reinhold, D., Kunz, D., Schulz, H.U., Schneemilch, C., Brandt, W., and Ansorge, S. (**1995**). Effects of interleukin-6 (IL-6) and transforming growth factor-beta (TGF-beta) on neutrophil elastase release. *Inflammation* **19**(1): p. 83-99.

227. Aldridge, A.J. (**2002**). Role of the neutrophil in septic shock and the adult respiratory distress syndrome. *The European Journal of Surgery* **168**(4): p. 204-14.

228. Raghavendran, K., Nemzek, J., Napolitano, L.M., and Knight, P.R. (**2011**). Aspiration-induced lung injury. *Critical Care Medicine* **39**(4): p. 818-26.

229. Zaloga, G.P. (**2002**). Aspiration-related illnesses: definitions and diagnosis. *Jounal of Parenteral Enteral Nutrition* **26**(6 Suppl): p. S2-7; discussion S7-8.

230. Kennedy, T.P., Johnson, K.J., Kunkel, R.G., Ward, P.A., Knight, P.R., and Finch, J.S. (**1989**). Acute acid aspiration lung injury in the rat: biphasic pathogenesis. *Anesthesia and Analgesia* **69**(1): p. 87-92.

231. Marik, P.E. (**2001**). Aspiration pneumonitis and aspiration pneumonia. *The New England Journal of Medicine* **344**(9): p. 665-71.

232. Olsson, G.L., Hallen, B., and Hambraeus-Jonzon, K. (**1986**). Aspiration during anaesthesia: a computer-aided study of 185,358 anaesthetics. *Acta Anaesthesiologica Scandinavica* **30**(1): p. 84-92.

233. Warner, M.A., Warner, M.E., and Weber, J.G. (**1993**). Clinical significance of pulmonary aspiration during the perioperative period. *Anesthesiology* **78**(1): p. 56-62.

234. Bodlander, F.M. (**1975**). Deaths associated with anaesthesia. *British Journal of Anaesthesia* **47**(1): p. 36-40.

235. Goldman, G., Welbourn, R., Kobzik, L., Valeri, C.R., Shepro, D., and Hechtman, H.B. (**1992**). Synergism between leukotriene B4 and thromboxane A2 in mediating acid-aspiration injury. *Surgery* **111**(1): p. 55-61.

236. Nagase, T., Ohga, E., Sudo, E., Katayama, H., Uejima, Y., Matsuse, T., and Fukuchi, Y. (**1996**). Intercellular adhesion molecule-1 mediates acid aspiration-induced lung injury. *American Journal of Respiratory and Critical Care Medicine* **154**(2): p. 504-510.

237. Davidson, B.A., Knight, P.R., Helinski, J.D., Nader, N.D., Shanley, T.P., and Johnson, K.J. (**1999**). The role of tumor necrosis factor-alpha in the

pathogenesis of aspiration pneumonitis in rats. *Anesthesiology* **91**(2): p. 486-99.

238. Knight, P.R., Davidson, B.A., Nader, N.D., Helinski, J.D., Marschke, C.J., Russo, T.A., Hutson, A.D., Notter, R.H., and Holm, B.A. (**2004**). Progressive, severe lung injury secondary to the interaction of insults in gastric aspiration. *Experimental Lung Research* **30**(7): p. 535-57.

239. Raghavendran, K., Davidson, B.A., Mullan, B.A., Hutson, A.D., Russo, T.A., Manderscheid, P.A., Woytash, J.A., Holm, B.A., Notter, R.H., and Knight, P.R. (**2005**). Acid and particulate-induced aspiration lung injury in mice: importance of MCP-1. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **289**(1): p. L134-43.

240. Shanley, T.P., Davidson, B.A., Nader, N.D., Bless, N., Vasi, N., Ward, P.A., Johnson, K.J., and Knight, P.R. (**2000**). Role of macrophage inflammatory protein-2 in aspiration-induced lung injury. *Critical Care Medicine* **28**(7): p. 2437-44.

241. Kudoh, I., Ohtake, M., Nishizawa, H., Kurahashi, K., Hattori, S., Okumura, F., Pittet, J.F., and Wiener-Kronish, J. (**1995**). The effect of pentoxifylline on acid-induced alveolar epithelial injury. *Anesthesiology* **82**(2): p. 531-41.

242. Goldman, G., Welbourn, R., Kobzik, L., Valeri, C.R., Shepro, D., and Hechtman, H.B. (**1990**). Tumor necrosis factor-alpha mediates acid aspiration-induced systemic organ injury. *Annals of Surgery* **212**(4): p. 513-9; discussion 519-20.

243. Goldman, G., Welbourn, R., Kobzik, L., Valeri, C.R., Shepro, D., and Hechtman, H.B. (**1992**). Reactive oxygen species and elastase mediate lung permeability after acid aspiration. *Journal of Applied Physiology* **73**(2): p. 571-5.

244. Davidson, B.A., Knight, P.R., Wang, Z., Chess, P.R., Holm, B.A., Russo, T.A., Hutson, A., and Notter, R.H. (**2005**). Surfactant alterations in acute inflammatory lung injury from aspiration of acid and gastric particulates. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **288**(4): p. L699-708.

245. Goldman, G., Welbourn, R., Klausner, J.M., Kobzik, L., Valeri, C.R., Shepro, D., and Hechtman, H.B. (**1991**). Neutrophil accumulations due to pulmonary thromboxane synthesis mediate acid aspiration injury. *Journal of Applied Physiology* **70**(4): p. 1511-7.

246. Tsuchida, S. and Kavanagh, B.P. (**2014**). Ventilator-induced lung injury, in *Pediatric Critical Care Medicine: Volume 2: Respiratory, Cardiovascular and Central Nervous Systems*, Wheeler, D.S., Wong, H.R., and Shanley, T.P., Editors. Springer London: London. p. 237-248.

247. Allen, G.B., Leclair, T.R., von Reyn, J., Larrabee, Y.C., Cloutier, M.E., Irvin, C.G., and Bates, J.H. (**2009**). Acid aspiration-induced airways hyperresponsiveness in mice. *Journal of Applied Physiology* **107**(6): p. 1763-70.

248. Folkesson, H.G., Matthay, M.A., Hebert, C.A., and Broaddus, V.C. (**1995**). Acid aspiration-induced lung injury in rabbits is mediated by interleukin-8-dependent mechanisms. *The Journal of Clinical Investigation* **96**(1): p. 107-16.

249. Sohma, A., Brampton, W.J., Dunnill, M.S., and Sykes, M.K. (**1992**). Effect of ventilation with positive end-expiratory pressure on the development of lung damage in experimental acid aspiration pneumonia in the rabbit. *Intensive Care Medicine* **18**(2): p. 112-117.

250. Pourfathi, M., Cereda, M., Chatterjee, S., Xin, Y., Kadlecek, S., Duncan, I., Hamedani, H., Siddiqui, S., Profka, H., Ehrich, J., Ruppert, K., and Rizi, R.R. (**2018**). Lung metabolism and inflammation during mechanical ventilation; an imaging agpproach. *Scientific Reports* **8**(1): p. 3525.

251. van Westerloo, D.J., Knapp, S., van't Veer, C., Buurman, W.A., de Vos, A.F., Florquin, S., and van der Poll, T. (**2005**). Aspiration pneumonitis primes the host for an exaggerated inflammatory response during pneumonia. *Critical Care Medicine* **33**(8): p. 1770-8.

252. Rotta, A.T., Shiley, K.T., Davidson, B.A., Helinski, J.D., Russo, T.A., and Knight, P.R. (**2004**). Gastric acid and particulate aspiration injury inhibits pulmonary bacterial clearance. *Critical Care Medicine* **32**(3): p. 747-54.

253. Frank, J.A., Gutierrez, J.A., Jones, K.D., Allen, L., Dobbs, L., and Matthay, M.A. (**2002**). Low tidal volume reduces epithelial and endothelial injury in acid-injured rat lungs. *American Journal of Respiratory and Critical Care Medicine* **165**(2): p. 242-9.

254. Walker, M.G., Yao, L.J., Patterson, E.K., Joseph, M.G., Cepinskas, G., Veldhuizen, R.A., Lewis, J.F., and Yamashita, C.M. (**2011**). The effect of tidal volume on systemic inflammation in Acid-induced lung injury. *Respiration* **81**(4): p. 333-42.

255. Kawamae, K., Pristine, G., Chiumello, D., Tremblay, L.N., and Slutsky, A.S. (**2000**). Partial liquid ventilation decreases serum tumor necrosis factor-alpha concentrations in a rat acid aspiration lung injury model. *Critical Care Medicine* **28**(2): p. 479-83.

256. Gurkan, O.U., O'Donnell, C., Brower, R., Ruckdeschel, E., and Becker, P.M. (**2003**). Differential effects of mechanical ventilatory strategy on lung injury

and systemic organ inflammation in mice. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **285**(3): p. L710-8.

257. Bachofen, H., Schürch, S., Michel, R.P., and Weibel, E.R. (**1993**). Experimental hydrostatic pulmonary edema in rabbit lungs. *American Review of Respiratory Disease* **147**(4): p. 989-996.

258. Nguyen, T.L. and Perlman, C.E. (**2018**). Tracheal acid or surfactant instillation raises alveolar surface tension. *Journal of Applied Physiology* **125**(5): p. 1357-1367.

259. Tiret, L., Desmonts, J.M., Hatton, F., and Vourc'h, G. (**1986**). Complications associated with anaesthesia--a prospective survey in France. *Canadian Anaesthetists Society Journal* **33**(3 Pt 1): p. 336-44.

260. Holland, R. (**1984**). Anesthesia-related mortality in Australia. *International Anesthesiology Clinics* **22**(2): p. 61-71.

261. Arbous, M.S., Grobbee, D.E., van Kleef, J.W., de Lange, J.J., Spoormans, H.H., Touw, P., Werner, F.M., and Meursing, A.E. (**2001**). Mortality associated with anaesthesia: a qualitative analysis to identify risk factors. *Anaesthesia* **56**(12): p. 1141-53.

262. Lellouche, F., Dionne, S., Simard, S., Bussieres, J., and Dagenais, F. (**2012**). High tidal volumes in mechanically ventilated patients increase organ dysfunction after cardiac surgery. *Anesthesiology* **116**(5): p. 1072-82.

263. Hedenstierna, G. and Edmark, L. (**2010**). Mechanisms of atelectasis in the perioperative period. *Best Practice and Research Clinical Anaesthesiology* **24**(2): p. 157-69.

264. Martinez, G. and Cruz, P. (**2008**). [Atelectasis in general anesthesia and alveolar recruitment strategies]. *Revista Española de Anestesióloga y Reanimación* **55**(8): p. 493-503.

265. Levin, M.A., McCormick, P.J., Lin, H.M., Hosseinian, L., and Fischer, G.W. (**2014**). Low intraoperative tidal volume ventilation with minimal PEEP is associated with increased mortality. *British Journal of Anaesthesia* **113**(1): p. 97-108.

266. Guay, J. and Ochroch, E.A. (**2015**). Intraoperative use of low volume ventilation to decrease postoperative mortality, mechanical ventilation, lengths of stay and lung injury in patients without acute lung injury. *The Cochrane Database of Systematic Reviews* (12): p. Cd011151.

267. Deans, K.J., Minneci, P.C., Cui, X., Banks, S.M., Natanson, C., and Eichacker, P.Q. (**2005**). Mechanical ventilation in ARDS: One size does not fit all. *Critical Care Medicine* **33**(5): p. 1141-3.

268. Eichacker, P.Q., Gerstenberger, E.P., Banks, S.M., Cui, X., and Natanson, C. (**2002**). Meta-analysis of acute lung injury and acute respiratory distress syndrome trials testing low tidal volumes. *American Journal of Respiratory and Critical Care Medicine* **166**(11): p. 1510-4.

269. Parhar, K.K.S., Zjadewicz, K., Soo, A., Sutton, A., Zjadewicz, M., Doig, L., Lam, C., Ferland, A., Niven, D.J., Fiest, K.M., Stelfox, H.T., and Doig, C.J. (**2019**). Epidemiology, Mechanical Power, and 3-Year Outcomes in Acute Respiratory Distress Syndrome Patients Using Standardized Screening. An Observational Cohort Study. *Annals of the American Thoracic Society* **16**(10): p. 1263-1272.

270. Nieman, G.F., Satalin, J., Andrews, P., Aiash, H., Habashi, N.M., and Gatto, L.A. (**2017**). Personalizing mechanical ventilation according to physiologic parameters to stabilize alveoli and minimize ventilator induced lung injury (VILI). *Intensive Care Medicine Experimental* **5**(1).

271. Nieman, G., Satalin, J., Andrews, P., Wilcox, K., Aiash, H., Baker, S., Kollisch-Singule, M., Madden, M., Gatto, L., and Habashi, N. (**2018**). Preemptive mechanical ventilation based on dynamic physiology in the alveolar microenvironment: Novel considerations of time-dependent properties of the respiratory system. *The Journal of Trauma and Acute Care Surgery* **85**(6): p. 1081-1091.